VOLUME 2 MAY 1956 NUMBER 3

Canadian Journal of Microbiology

This supplementary issue
is dedicated to
PROFESSOR E. G. D. MURRAY
in recognition of his contribution
to science

Published by THE NATIONAL RESEARCH COUNCIL
OTTAWA CANADA

CANADIAN JOURNAL OF MICROBIOLOGY

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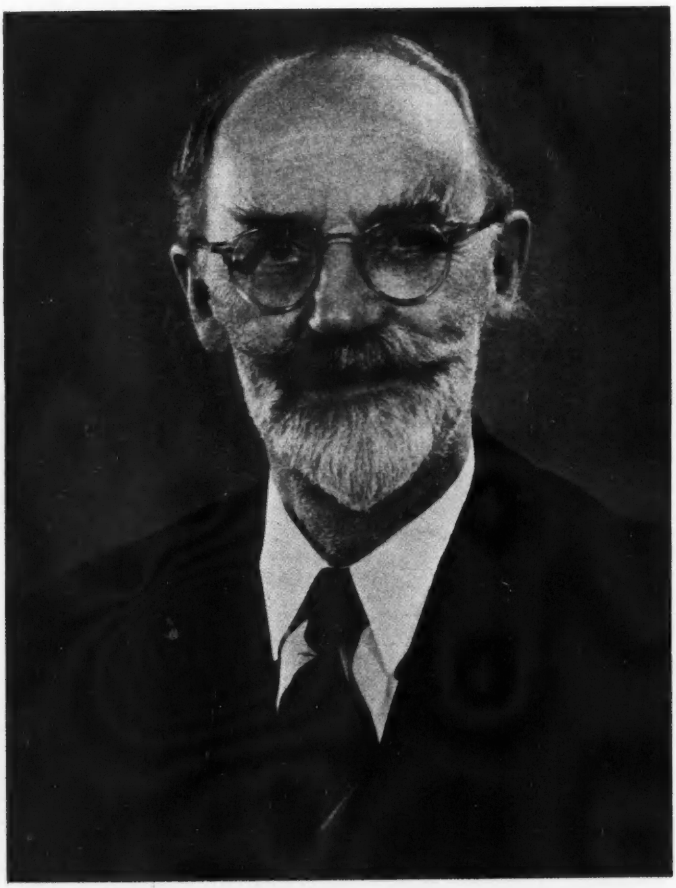

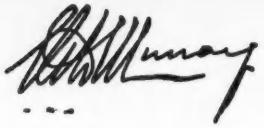

FOREWORD

This issue of the Canadian Journal of Microbiology pays respect to a man who is known to some as "Joburg", to others as the "Chief", and to many as Professor Murray. To some he has been a teacher, to some a counsellor, and to some a stern critic; but to all he has been a friend. He is a scientist by nature and a bacteriologist by profession; he is a fisherman by inclination and a raconteur with few equals.

The papers in this issue are tokens of esteem from his pupils, his colleagues, and his friends. Many have contributed, and many others wished to do so. All feel that these tributes should be offered to him while he is in the prime of his retirement. All hope that "E.G.D." will derive pleasure from them.

It was especially gratifying to receive a communication from Professor H. R. Dean of Cambridge University under whom E. G. D. Murray trained and worked. Professor Dean's personal tribute has been included in the "Appreciation".

The papers are not in any particular sequence except for the first few which are general in nature. These review some of the changes in Bacteriology that have come about in Dr. Murray's lifetime or deal with topics that have been prominent in his interests.

It has been our privilege to share in the preparation of this issue of the Journal. We join his many friends in extending to him our affection and sincere good wishes.

T. E. Roy
A. A. Miles

Professor E. G. D. MURRAY

An Appreciation[1]

Everitt George Dunne Murray, O.B.E., M.A. (Cantab.), L.M.S.S.A., D.Sc. (Montreal), D.Sc. (McGill), F.R.S.C., is a scholar, educator, researcher, bibliophile, antiquarian, "naturalist" in the real tradition of the old school, and a lover of the great out-of-doors.

We are greatly pleased to pay him tribute on the occasion of his retirement from the Chair of Bacteriology and Immunology at McGill University. We are greatly pleased to honor him because as Canadian Microbiologists, we have been privileged for twenty-five years to learn from one whose keen and fertile mind is always conscious of the "subtle relations existent between things". As members of his staff at McGill, we are sad because we shall no longer be as near to him as we once were. But knowing his vigor of mind and body, we are gratified that we have not heard the last from him despite his departure from McGill.

E. G. D. Murray is of that generation which was heir to the thinking of the Microbiologists of the last century. His training and intellectual attitudes were grounded first in the other sciences, and his breadth of experience coupled with an astute perception of the interrelationships in biology and medicine set the pitch and tone for the thinking and the work of those close about him. His high ideals were often difficult for others to attain, but for all, he has always set up barriers against entry into the "dead-end streets" of self-sufficiency and isolation in Microbiology.

Professor Murray was born in Johannesburg, South Africa, in 1890. Though a respecter of many solid traditions, he was permanently stamped with the independence characteristic of the pioneers of his native land, an independence sometimes approaching that of the rebel. More than this, he has the energy of the pioneer. All who have known him sense these things well. He is an explorer, a builder, and an investor in talent.

From South Africa, he went to England to receive his formal education at Christ's College, Cambridge, and his medical training at "Bart's" in London. By 1918 he had won his B.A., M.A., and Licentiate in Medicine and Surgery. During this time the First Great War was in progress, and, as Captain E.G.D. Murray of the Royal Army Medical Corps, he was given special duties with the Research Staff of the War Office Control Laboratory for Cerebro-Spinal Fever, was sent on a War Office Mission to the Pasteur Institute for investigations of *Meningococcus* antiserum, and went to Mesopotamia to work on dysentery. In Mesopotamia, he contracted dysentery and was invalided to India. On his return to England, he was assigned to the Staff of the War Office Vaccine Department where he worked from 1917 to 1919. He also sat

[1]*Contribution from the staff of the Department of Bacteriology and Immunology, McGill University, Montreal, Quebec.*

as one of the British Representatives to the Allied Medical Services Conference in Paris in 1917, and was made a member of the War Office Commission on Dysentery in 1918. In recognition of his war work he was awarded the O.B.E. (Military Division) in 1918.

His activities during the war were the prelude to the next twelve years of work in England which were characterized by a tremendous flow of energy and a dedicated will to accomplish difficult and demanding tasks. He returned to Cambridge University where he became known fondly to his many friends as "Joburg". From 1920 to 1926 he was Research Bacteriologist and Member of the Committee on *Meningococcus* and *Pneumococcus* Serum of the Medical Research Council of Great Britain. The results of his extensive investigations of the *Meningococcus* were published in a Monograph which remains an authoritative work to this day. His academic and administrative achievements at Cambridge attest to his unusual abilities while still a young man. He was, between 1923 and 1930, a Fellow of Christ's College and Lecturer in Pathology and Director of Medical Studies at Christ's College. During this time he was active as a Member of the Board of Biology, the Board of Medicine, the Degrees Committee Board of Biology, the University Building Syndicate, and he sat as Examiner in the Natural Sciences Tripos and in Pathology in the Faculty of Medicine. His activities were great and varied, and as Professor H. R. Dean, who is Head of the Department of Pathology at the University of Cambridge, has written recently, "In the prosecution of research he was indefatigable, and we were proud to know that he expected from his friends a standard of industry equal almost to his own. He was always ready to help a colleague, and his comments and advice were awaited with an interest enhanced always by an unexpected phraseology. Murray played a big part in the development of our teaching programme. His election to the Chair of Bacteriology in McGill University brought pride to the hearts of his Cambridge colleagues but sorrow at the loss of a much loved friend".

E. G. D. Murray is a "builder", as we have said, and on leaving Cambridge in 1930 to come to Canada, he left behind him not only many friends and many memories but also a building. Before leaving he had the pleasure of seeing his love for structure and equipment materialize in the new Department of Pathology at Cambridge. To quote Professor H. R. Dean again, "In the preparation of the plans for the new building, Murray threw himself with all his customary energy and enthusiasm, and now, after twenty-seven years, there are still features and details of our laboratory which remind us of our old friend and colleague, a memorial not unworthy of his skill and foresight, for Murray has never been satisfied with anything but the best." We at McGill have heard in most colorful words of the various "altercations" that arose during the planning and erection of the Pathology Laboratory in Cambridge. We have little doubt that there are still unexplored areas of the building where testimonials of his displeasure may be found. These may at any time be uncovered behind bits of molding or under an as yet undisturbed

wall panel. It will surely create an astonishing impression in some unapprised soul who finds, when engaged in the changing of a fuse and unaware of the permanency of history, the yellowed but veritable mate of an earlier discovery: "This damn thing is in the wrong place—E. G. D. Murray!".

Dr. Murray came to Canada in 1930 to assume the Professorship and Chairmanship of the Department of Bacteriology in the Faculty of Medicine at McGill University. One can perhaps appreciate some of his feelings, when, upon his arrival at McGill, he discovered that he was the sole member of his Department and that he was to have the assistance of one trained technician. He surveyed his new inheritance—a large bare laboratory with chemical benches and a half-dozen rooms with little or no equipment. The situation would have been most discouraging to any but a born pioneer. Before winter set in, Professor Murray had made available adequate facilities for the teaching of medical students. Before winter yielded to spring, the "Chief", and his new, full, bristling, and usually well-trimmed beard, was marked as a man to be reckoned with in the years ahead at McGill.

He was again a builder. During his twenty-five years at McGill, Professor Murray has rebuilt the Department in more ways than one. The physical facilities have been remodeled, enlarged, rebuilt, and re-equipped. The staff of his Department grew to nearly sixty persons, and his Department of Bacteriology and Immunology became recognized as one of the leading centers in Canadian Microbiology. The Department developed along diverse but related lines—teaching, graduate study, research, and diagnostic medical bacteriology. His dictum that teaching in Bacteriology should be provided wherever and whenever the need arose has led to a close liaison between his Department and many Faculties of the University during his tenure of office. With his personal participation in all courses, instruction has been given in the fields of Medicine, Public Health, Nursing, Dentistry, and Arts and Science with separate and appropriate courses for each. Graduate studies became a particular interest and at the time of his retirement there were fifteen postgraduate students occupied in various stages of their research toward M.Sc. or Ph.D. degrees. Even during the brief period since the last war, some forty-four postgraduate students from his Department have distributed themselves across Canada or the United States in university, government, hospital, or industrial laboratories. It is true to say that many of the major teaching hospitals in Montreal, in the rest of Canada, and some in the United States have been or are staffed by professional and technical personnel who have had the benefit of his teaching.

His great energy and persistence in the pursuit of objectives which he believed to be his responsibility to achieve have been rewarded by the increasing interest and recognition given to Bacteriology and Immunology by the medical profession in Montreal. His active interest in the problems of diagnostic and applied bacteriology and his experienced judgment have been of immense benefit to clinical medicine in at least seven metropolitan hospitals in Montreal and its environs. He has been Bacteriologist-in-Chief and a Member of the Medical Boards of the Royal Victoria Hospital and of the

Children's Memorial Hospital as well as a member of the Medical Boards of both the Royal Edward Laurentian and Alexandra Hospitals. Within McGill University, he was a Member of the Faculties of Medicine, Arts and Science, and Graduate Studies, and sat in the University Senate from 1943 to 1953 as an Elected Representative of the Faculty of Medicine. From 1949 until his retirement from McGill he served as one of the two University representatives in the City Council of Montreal as a Class C Councillor.

As the years went by there were increasing demands made upon E. G. D. Murray's time by bodies in Canada, in the United States, and elsewhere. All have profited by his broad interests and experience and by his energy of mind. He holds membership in many scientific societies in which he served often in an executive capacity. As well as his active association with older national and international scientific groups, he has given enthusiastic and unstinting support to fledgling organizations, in which one must include the Canadian Society of Microbiologists.

Dr. Murray's interest in Bacterial Taxonomy and Nomenclature was recognized in 1933 when he became a member of the Committee on *Bergey's Manual of Determinative Bacteriology*, and later in 1939 when he was appointed to the Board of Editor-Trustees. Since 1940 he has been an Associate Editor of the American Journal of Medical Sciences, Section of "Progress of Medical Science, Pathology and Bacteriology".

Except for the years of the Second World War, the "Chief" never left his Department for any prolonged length of time, but his activities in the war years necessitated frequent absences. Between 1940 and 1945 he was a member of the Subcommittees of the National Research Council on Infections and Shock and Blood Substitutes as well as of the Wartime Prices and Trade Board Pharmaceutical Advisory Committee. He was the Canadian Chairman of the Joint United States – Canadian Commission at the War Diseases Control Station, Chairman of the Biological Warfare Committee, and a Superintendent of Research in the Directorate of Chemical Warfare and Smoke at National Defence Headquarters in Ottawa. Later, from 1946 to 1948, he remained as a Member of an Advisory Board and as Chairman of a Panel under the Defence Research Board of Canada. He has been associated with the National Research Council in several capacities since 1947, and in 1953 he was honored by appointment to the Honorary Advisory Council of the National Research Council.

Professor Murray throughout his professional life has contributed regularly but always critically to Microbiology by his scientific publications, by his lectures before learned societies, and by his keen comments on papers being read before meetings of various Associations. These comments were sometimes caustic, but always well-deserved, constructive, and sound. His publications on the *Meningococcus*, the dysentery bacilli, the *Listeria*, and the *Spirochaetaceae* all attest to his broad interests. Bacterial taxonomy and nomenclature, immunity, and immunity reactions have received considerable attention in his published works. His domains have included Public Health,

Veterinary Medicine, and others. In the later years, he has concerned himself with "constructive speculation", a field for which he is eminently suited because of his wide experience, critical judgment, and originality of thought. The titles of some of his more recent publications indicate something of this—"The Progress of Medicine", "Public Influences in the Medical Profession", "Why be a Microbiologist?", "Nature's Place in Man's World". His new duties as Honorary Professor in the Department of Medical Research at the University of Western Ontario are designed to provide the opportunity for him to make as valuable contributions in the future as he has in the past. We know that we shall reap more from his fertile and productive mind through the written and spoken word.

Only at the actual time of his leaving McGill did we become fully aware of a feeling of personal loss. During the last few years, even though his outside activities had been making greater and greater inroads into his time and energies, his hand never left the helm at McGill. He has not been slackening his pace with the approach of retirement, but to his last day in the Department he has responded vigorously to the challenge of each routine daily task. On the very day he donned his hat and said "Goodbye", he noted that a new air circulator had been incorrectly installed during the previous week end. He remarked with vigor that this mishap would surely seed our plates with some interesting contaminants this winter when the circulator was turned on, and decried in appropriately colorful language the blind-minded idea that one can circulate "Hot" air without circulating "what's in it".

E. G. D. Murray has long been honored and respected among his colleagues, associates, and students, but it is most gratifying to know that his attributes of mind and character have received wide recognition. He was awarded the O.B.E. (Military Division) in 1918 and made a Fellow of the Royal Society of Canada in 1938, but it is particularly since the last war that public honor has been given him. He was awarded the Medal of Freedom in 1947 by the United States War Department. The Flavelle Medal from the Royal Society of Canada was awarded him in 1953. In May of 1953, Professor Murray was pleased to receive the Coronation Medal of Queen Elizabeth II. At the Spring Convocation in 1955 the University of Montreal presented him with the honorary degree of Doctor of Medical Science and made him a Member of the Executive Council of the Institut de Microbiologie of the University. McGill University, in October 1955, honored him with the degree of Doctor of Science.

We, of his staff at McGill, join in the public recognition of Professor Murray's contributions to Science, but we hold a quite personal and affectionate regard for the "Chief" which is part of and yet distinct from the man of science. We know of small groups who will never forget his support and understanding: those newly-arrived in Canada, those struggling with administrative and organizational frustrations behind the scenes, and the discouraged but, to E. G. D. Murray's mind, worthwhile students. We hope we have learned well his credo—that intellectual sloth is anathema, that members of his "official family" must share in the knowledge of current research at home and abroad.

We know him as the stimulating enthusiast, the "ferment" who has kept the brew brewing. Positive in criticism, his attacks came not as the repeated barbs of a persistent small creature but as the judicious laying-on of a bull whip. If one has the hide to withstand it, and only a worthwhile hide will withstand it, one enjoys the advantages of a full education of the mind under his tutelage. Never known to us as a solemn man, the "Chief" engendered real but affectionate respect for his authority and judgment in all who have been fortunate to be at some time members of his "official family". Never loath to express criticism where needed, he never withheld praise when it was merited, even from the unseasoned. He has been a mentor, an inspiration, and a beloved paternal comrade. He shall be sorely missed.

Canadian
Journal of Microbiology

Issued by THE NATIONAL RESEARCH COUNCIL OF CANADA

VOLUME 2 MAY 1956 NUMBER 3

PROGRESS IN MEDICAL BACTERIOLOGY, 1915–1955[1]

BY L. P. GARROD

Abstract

The main advances in medical bacteriology during the past forty years are reviewed under six headings. Some new species have been discovered, but much more has been done in subdividing existing species with benefit to therapeutics and epidemiology. The chief advances in technique have been the introduction of electron microscopy and of many highly selective culture media. The study of bacterial metabolism has grown into a new branch of the science, and has provided a rational approach to new forms of chemotherapy. There has been little advance in the understanding of immunity, but methods of immunization have been greatly improved and extended. Finally, the discovery of the therapeutic action first of the sulphonamides and then of the antibiotics has not only revolutionzed therapeutics but given a new direction to laboratory work, and increased both its volume and its complexity.

The forty years of Murray's career as a bacteriologist have seen an immense development of the science to which he devoted his life. This succeeded a period of comparative stagnation in the early years of the century, and the first incentive to progress was the War of 1914–18 and the imperative demands made by it in relation to wound sepsis and epidemic disease. An increasing general awareness of the contributions which bacteriology can make to medicine in almost all its branches, and greatly increased facilities and support for research, have since then produced a steady expansion: there are probably five times as many people doing bacteriological work as there were forty years ago, and perhaps twenty times as many doing nothing else and well enough paid for it to be able to follow their own bent. The result of the efforts of some of these people has been to transform the whole face of medicine.

Perhaps the greatest achievements have been the almost complete disappearance of diphtheria in many countries owing to immunization, and the reduction in mortality from acute coccal infections which has been brought about by chemotherapy. These typify advances in two branches of the science: in all there seem to be six which call for separate consideration if a true picture of events during this period is to be given.

[1]*Manuscript received October 29, 1955.*
Contribution from St. Bartholomew's Hospital, London, United Kingdom.

[The April number of this Journal (Can. J. Microbiol. 2 : 65-138. 1956) was issued April 6, 1956.]

The Recognition of New Species

The first recognition of species of *Leptospira* and of *Rickettsia* dates from exactly the beginning of this period, and impetus was given to the study of both Weil's disease and typhus by their occurrence under war conditions, the former particularly in trenches in France. The first species of *Bartonella* and *Spirillum minus* were also discovered at about this time. *Listerella monocytogenes* was described by Murray himself and others in 1926. Various new *Mycobacteria* have come to light, of which the vole bacillus, discovered by Wells in 1937, has been the most significant in practical consequences. The year 1917 will be remembered for the simultaneous discovery by Twort and d'Herelle of bacteriophage. This account could do no justice to its subject if it were to stray beyond the confines of bacteriology in the stricter sense of that word and embrace virology, almost the whole development of which into the subject it is today has taken place in well under forty years. The number of new viruses recognized must considerably exceed that of all other new pathogenic microorganisms.

If few positively new bacteria have been discovered, much has been done by way of broadening knowledge of imperfectly recognized species within certain genera. Both World Wars gave an impetus to the study of organisms responsible for gas gangrene, and much of our present knowledge of individual clostridia dates from 1914–18. This was largely owing to the works of British and French bacteriologists, who found abundant material in the appalling numbers of casualties on the Western Front. Later developments have been concerned less with the identification of species, some of which probably play little or no part in producing true clostridial myositis as distinct from ischaemic gangrene, and more with defining the conditions under which the former develops and with studying the toxins of the few species mainly responsible. Attention during 1939–45 and since has been concentrated largely on the many toxins of *Clostridium welchii*, but the mode of action of those of Type A in producing systemic effects is still imperfectly understood.

It is not often that a disease is recognized as a clinical entity with a known cause 30 years after that cause has been discovered in another environment. For the recognition of *Brucella abortus* as a human pathogen and a near relative of *Brucella melitensis* we have to thank Alice Evans, and although she pointed out this relationship in 1918, it was not until about ten years later that undulant fever due to *B. abortus* began to be generally recognized and diagnosed in England and elsewhere. The study of "brucellosis" in all its forms apart from the long familiar Malta fever is a development wholly within this period.

By far the greatest expansion in the number of recognized pathogenic species within a genus has been among the salmonellas. The work of Andrewes, and later particularly of Bruce White and Kauffmann, has made it possible to identify over two hundred organisms responsible for infective enteritis, a number to which additions are still being made. This is really an example of serological classification, to which further reference will be made, and these named so-called species have no more claim to specific rank than numbered

serological types of streptococci and pneumococci, but the elucidation of the complex antigenic structure of the salmonellas is nevertheless an outstanding achievement of the time. A similar but much less extensive expansion has taken place in the number of recognized types of *Shigella*.

The Identification of Types within a Species by Serological and Other Methods

The beginning of this period saw the continuation by Dochez and Avery of work initiated by Neufeld and Händel on the serological classification of pneumococci. The study of pneumonia concerned itself with the three types identified by these workers until the early 'thirties', when Cooper defined 29 further types among hitherto unclassified strains: the list has since been even further extended by Lund. The motive of these and other efforts at this period was therapeutic: only by type ascertainment and the use of monotypical antiserum was it possible to furnish effective specific treatment for a disease causing more deaths by far than any other acute bacterial infection. This motive, accentuated by the epidemic prevalence of cerebrospinal fever among British troops, inspired Gordon and Murray himself in work which led to the definition of four serological types of meningococcus.

The study by similar methods of haemolytic streptococci has had practical consequences of a quite different kind. The ascertainment of its Lancefield group makes it possible to assign a streptococcus to one of a series of broad categories, ecologically and pathogenically distinct. The value of this proceeding in assessing the significance of the individual carrier state has alone been immeasurable. Efforts by many workers to classify the streptococci of human infections—i.e., with few exceptions those within Lancefield's Group A—culminated in the definition by Griffith of about thirty types distinguishable by agglutination. The applications of this method have been epidemiological, and the consequences to our knowledge of how infection is spread have been far-reaching: for mere conjecture in connection with hospital cross-infection, with sources of infection in puerperal fever, and with the origins of milk-borne and other epidemics of scarlet fever, we have been able to substitute certainty and fact. Griffiths' work was the foundation on which a major advance in hygiene has been built.

The method of bacteriophage typing introduced by Craigie and Yen has been resorted to for the same purpose in dealing with organisms of constant antigenic constitution and therefore incapable of subclassification by serological methods. Some extraordinary pieces of detective work have been made possible by this method in connection with episodes of typhoid fever. More recently phage typing has been applied to staphylococci with consequences to the study of cross-infection very similar to those made possible by the Griffith typing of streptococci: perhaps its most singular achievements have been in tracing the occult and trivial sources of outbreaks of staphylococcal food poisoning.

There has been one outstanding example of the use of a much older method in defining types within a species. McLeod's classification of *Corynebacterium diphtheriae* depends simply on cultural characters, and until this was put forward it might well have been supposed that the possibilities of this form of study had long before been exhausted.

These have been the major achievements of type definition by one method or another. It may be added that most of the other important pathogens have now been examined with a view to defining serological types within them, with frequently positive but in no instance such significant results.

Improvements in Technique

Much of the day-to-day work of a bacteriological laboratory is carried out by the same simple methods as were in use forty years ago. It could perhaps be more highly mechanized with advantages in timesaving, but with loss in another direction, for there can be few sciences in which manipulative skill and the fruits of long experience make so much difference to the quality of work. On the other hand there have been major advances in specialized methods both for studying bacterial morphology and for cultivation.

Ordinary microscopy has improved little, and neither fluorescence nor phase contrast microscopy—each an innovation within this period—has conferred any major benefits on the science of bacteriology. The electron microscope has been another matter: it has revealed details in the structure of the bacterial cell, and of the changes wrought in it by external agencies, which are awe-inspiring in the clarity with which they can be depicted at immense magnifications.

Methods of cultivation have been improved mainly by the introduction of highly selective media. Perhaps the ingenuity of those responsible for such media has been most fruitful in the isolation of intestinal pathogens, which is now conducted with a precision and certainty positively embarrassing to the clinician in charge of a persistent carrier or to the Medical Officer of Health in whose area are warehouses containing tons of imported dried egg products. Penicillin, tellurite, brine, and gentian violet media have been of similar service for isolating respectively *Hemophilus pertussis*, *C. diphtheriae*, staphylococci from dust or faeces, and haemolytic streptococci from air or dust. The latest development of such methods is the use of antibiotics for the isolation of certain pathogenic fungi in culture and of viruses in the chick embryo.

The Study of Bacterial Metabolism

At the beginning of this period the choice of a culture medium for any purpose was purely empirical. It had been observed that complex natural materials of largely undefined composition, such as meat and vegetable extracts, mammalian body fluids, and egg, promoted satisfactory growth, and these were introduced without much further inquiry into the use made of them by bacteria. By means of synthetic, or at least chemically defined, media, it has now been possible to determine the growth requirements of different species and to follow

the processes by which nutrients are utilized. At the same time the biochemist has found in bacteria a convenient agency for rapidly effecting chemical changes in which he is interested, and what is almost a new science has grown up on the confines of bacteriology and biochemistry.

The more fundamental a line of advance, the more slowly will its consequences be apparent in any applied sphere. Perhaps the chief outcome of these studies so far has been in revealing the mode of action of certain antibacterial substances: the work of Fildes and Woods on the action of mercury salts and the sulphonamides formulated a new principle which has given direction to many subsequent studies in the field of chemotherapy.

Immunity and Immunization

My earliest recollection of Murray is his lectures to a small group of "pathological clerks" at St. Bartholomew's: although our interests at the time were strictly practical, a series of these lectures dealt exhaustively with theories of immunity, and were thus a corrective to any idea we may have had that laboratory diagnosis was the be-all and end-all of bacteriology. Ehrlich's side-chain theory and the law of Arrhenius and Madsen stand today almost where they did then, and little but the lattice theory of Marrack has been added to them. The study of individual antigen–antibody reactions *in vitro* has made much progress, and Landsteiner's work on haptenes has gone far to reveal their chemical basis. It must nevertheless be admitted that there has been little real advance in an understanding of the nature of immunity in the living body. Natural immunity—except in instances where differing temperature requirements of host and parasite afford us a facile explanation—remains a mystery. Unknown factors—apart, that is, from the state of resistance dependent on the possession of antibodies—evidently determine whether certain infections shall develop. We are similarly ignorant of the reasons why some infections persist for long or indefinitely, despite the demonstrable operation of the usual mechanisms for their elimination. Gonorrhoea typifies such infections: it is also an example of a disease in which the practical incentive to further study on immunological lines has been utterly removed. The conquests of chemotherapy have arrested potential progress in immunology.

While the study of immunity itself has been at a standstill, that of methods of immunization has advanced steadily in several directions and with immense benefit to health. Antitoxins have reached a high degree of refinement and potency, and it has been shown in lobar pneumonia that an antibacterial serum can influence the course of an acute infection to an extent not previously possible. The introduction of toxoid for active immunization against diphtheria in 1922 has led eventually to the virtual disappearance of this disease in many countries: tetanus toxoid, first used on any considerable scale in 1939, had a less easily distinguishable but similar effect.

The chief development in connection with killed bacterial vaccines has been the recognition that their prophylactic efficacy depends on the presence of antigens associated with virulence, and often lost in old cultures maintained

in the laboratory. It is a chastening thought that most of the evidence of the value of enteric fever prophylaxis dates from a time when this was unrecognized. That a properly prepared vaccine is a preventive of whooping cough has been proved conclusively only in quite recent years. Much the most important innovation has been BCG, which from unpromising and inconclusive beginnings has been developed to a point at which its use is almost world-wide and even the most sceptical have been forced to concede that it has at least some value.

Whole vaccines are crude products, and efforts have not been lacking to separate from them the antigen(s) exciting the immune response. This has been achieved to some extent with the pneumococcus, and a notable very recent advance has been the isolation in practically pure form of the protective antigen of *Bacillus anthracis*, a substance formerly known to exist only in the exudate of the disease itself.

Chemotherapy and the Concept of Antibiosis

The year 1956 marks the 21st anniversary of the public announcement that Prontosil (sulphamidochrysoidin) would overcome infection by haemolytic streptococci. This led within a year to the introduction of the first of the sulphonamides, to be followed by many others, with a steady broadening of the scope of their therapeutic action. The mortality from streptococcal sepsis of all kinds—notably puerperal fever—promptly fell to a small fraction of what it had been, and that of pneumonia followed, although to a less spectacular degree, since this infection is sometimes complicated by other grave conditions. That of cerebrospinal fever fell from about 50 to 5%, and gonorrhoea became rapidly and simply curable. In these and other conditions the bacteriologist met for the first time the difficulty of identifying pathogens which had suffered a crippling if not a knockout blow from treatment begun before his specimen was taken.

As if this revolution in therapy were not enough, the even more sensational properties of penicillin were revealed in 1941, and initiated a search for other antibiotics which, as it gathered momentum, assumed world-wide proportions, and formed the basis of a highly competitive and exceedingly prosperous new industry, centered largely in the United States. With about ten major antibiotics now available it is possible, with certain reservations, to treat successfully almost all acute bacterial infections, all rickettsial infections, and very few caused by the larger viruses. The generality of virus infections are still insusceptible, as are nearly all mycoses, an outstanding exception being actinomycosis.

This series of great discoveries has profoundly affected the work of the medical bacteriologist. It has in the first place rendered his diagnostic work often more difficult, and largely deprived him of a good deal of material useful for teaching which was formerly commonplace. It has also placed on him a new responsibility for the direction of treatment. He has not merely to identify the organism causing an infection, but to determine its sensitivity to

one or more antibiotics. On the skill and celerity with which this examination is conducted nowadays depends much of the value of the laboratory to the clinician. Because of the services he can so render, and for many contributions he has made to the prevention of infection, the bacteriologist has become the partner and adviser of the clinician instead of his servant.

Of the reservations made earlier with regard to the curative effect of antibiotics in bacterial infections generally, the most serious has to do with acquired bacterial resistance. The study of this phenomenon has assumed major importance in at least three directions: as it affects the individual patient, as it involves the community, and from the point of view of its mechanism. The third of these forms of study has been hampered by our comparative ignorance of how antibiotics act on the normally susceptible bacterial cell: it has also produced an interesting controversy on the respective parts played by adaptation and mutation in the acquisition of resistance. For the clinical bacteriologist these disturbing developments have brought new problems in determining the activity of two antibiotics applied together, since "combined" treatment may either be advisable to prevent acquired resistance or necessary to overcome it. Among other new techniques, those of antibiotic assay have their clinical uses as well as commercial and research applications.

Antibiosis, a term which seems appropriate when regarded as the antithesis of symbiosis, had in fact been studied in some of its manifestations for very many years before penicillin was discovered. With the impetus now given to its study, it stands clear as an important factor governing survival among crowded and varied microbic populations. Therapeutically useful antibiotics are only a few among thousands now known to exist, and owe their usefulness mainly to the accident of non-toxicity. In general an antibiotic is inimical only to unrelated species, but among many curiosities which have come to the light are the staphylococcines, substances which prevent the growth of other strains of the same organism. Not only staphylococci but streptococci, coliform bacilli, and clostridia have been shown to form substances inimical to the growth of other bacteria, and it is now at least strongly suspected that some of these substances operate *in vivo* to preserve a balance between different species in the human mouth and bowel. The removal of these restraints may be a partial explanation of "superinfections" by resistant staphylococci, fungi, and coliform bacilli following suppression of the normal flora by antibiotic treatment.

Other Developments

This period has seen an immense development of the study of allergy, not merely as a phenomenon accompanying infection, of disputed utility to the host, and incidentally of use in diagnosis, but as a possible cause of disease itself. It has seen the application of statistical methods to research, with an admirable corrective effect on previous sloppy thinking, although it sometimes seems that symbols and calculations of this kind are introduced rather because no paper is now considered complete without them than because they are

really needed. Wholly within this period has also been the introduction of a rational system of nomenclature for bacteria. This is a subject on which unanimity will never be achieved, and most people have their pet aversions among names for which others demand final acceptance. If I may be permitted to say so here, *Escherichia* is one of mine, not so much for its lack of euphony as because in its abbreviated form it becomes dangerously ambiguous: "*E. coli*" can equally well be *Entamoeba coli*, and the two organisms happen to be friends and neighbors in the same environment, and to be referred to frequently in the same document, namely, a report on the microbic content of faeces. I have also never understood why we should now be denied the distinctive and beautiful name *Staphylococcus*, redolent of vineyards in Greece, and be given a vague and characterless as well as clumsy and tripartite substitute.

Murray himself has played a large part in the orderly development of our nomenclature: this has indeed been one of the most valuable of his many contributions to the advancement of the science of bacteriology. I do not know how far he is responsible for my pet aversions, but in so far as he is I will try to forgive him, and I would do this for no other man.

QUALITATIVE DIFFERENCES IN THE IMMUNE RESPONSE[1]

By F. M. Burnet

Abstract

There are four types of response to an antigen: the production of classical antibody; the induction of hay fever type of hypersensitivity; the induction of tuberculin type of hypersensitivity; and the induction of specific immunological tolerance. Since the formation of antibody of some kind appears to be associated with the first three types of response, and the inhibition of antibody formation with immunological tolerance, any hypothesis of antibody formation must be consistent with what is known about all four responses. A reasonable consistency may be achieved by postulating the existence in the cell of endogenously produced 'genocopies' of certain proteins, which act as templates and determine the configuration of the proteins as they are synthesized; when a genocopy is modified by contact with antigen, protein will be synthesized with the specific complementary pattern of an antibody. The interference with this synthesis during embryonic life by entry into the cell of macromolecules objectionable to it, results in intracellular antibody and self-replicating mechanism for its further production—a system that can deal with all such macromolecules at the intracellular level; this corresponds to specific immune tolerance. When antigenic material enters body cells after birth, the same response occurs but there is in addition a transfer of the self-replicating mechanisms to cells of the lymphoid system with a consequent output of extracellular antibody—the classical antibody response. The hypersensitivity responses are intermediate in kind, and differ according to the transferability of the self-replicating mechanism, the amount of antibody released into the body fluids, and the readiness with which the antibody becomes attached to the tissue cells.

Introduction

It has become traditional since the days of Ehrlich and Landsteiner that immunology should be regarded primarily as the concern of the chemist. As long as attention is confined to the behavior of classical circulating antibody and its homologous antigen, it is possible to provide a very satisfying picture of the reactions concerned, along the lines made familiar by Pauling in his school. But immunology has untidy edges, it is a very biological subject that loses its essential quality if too closely circumscribed in mathematical and physicochemical concepts. This contribution in honor of one who has always had a biologist's approach to his subject is concerned with one of the untidy edges of immunology—the existence of at least four distinct types of immunological response.

Dissimilar Responses to the Same Antigen

Perhaps the best way to introduce the subject is to give some instances where one antigen can be shown to provoke two or more different types of immunological response. One of the most striking is the experience reported by Kuhns and Pappenheimer (17). Their interest was aroused by a child who, within a few minutes of a "booster" inoculation of diphtheria toxoid had a severe asthmatic reaction and whose skin was subsequently shown to react in typical immediate wheal and flare fashion to minute doses of highly purified

[1]*Manuscript received October 5, 1955.*
Contribution from the Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia.

diphtheria toxoid. The child's serum contained 50 units of antitoxin by rabbit skin titration, but showed no *in vitro* flocculation whatever. On further investigation, this type of response was found to be by no means uncommon amongst student volunteers. More detailed study of non-precipitating antitoxic serum from immunized subjects showed that the antibody was very similar to or identical with "hay fever type" antibody. Such an antitoxic serum injected intradermally will render a normal person's skin locally reactive (Prausnitz–Kustner reaction) and remains *in situ* and active for weeks. Heating at 56° for four hours removes the skin sensitizing power, but leaves its antitoxic titer untouched. The serum shows no Danysz effect, and fixes complement with toxoid only in the presence of a high concentration of serum. It does not precipitate with toxin or toxoid, but can be coprecipitated when antigen and normal precipitating antitoxic serum are added.

All these characters differ sharply from those of the classical diphtheria antitoxin produced in the majority of human subjects.

The second example is from the now classical work of Loveless (18), in which she differentiated the sensitizing antibody in the blood of hay fever patients from the blocking antibody, which in a number of persons at least was induced by the parenteral injection of pollen extracts. The blocking antibody had most of the qualities of a classical antibody and could be clearly differentiated from the sensitizing antibody by its heat stability and failure to transfer skin sensitization by the Prausnitz–Kustner method.

The existence of analogous differences in antibody produced in rabbits is indicated by Sherman, Menzel, and Seebohm (23). They immunized rabbits with ovalbumin, alone and precipitated with alum. Both produced good precipitating sera, but only those sera from animals immunized with alum-precipitated antigen conferred passive sensitization to human skin. By fractional precipitation of the serum with antigen, residual non-precipitating material was obtained, which still preserved the capacity to sensitize human skin. It would not be unjustifiable, therefore, to postulate that this portion of the antibody was of "hay fever type".

Tuberculin sensitivity is quite strikingly different from the sensitization associated with allergic conditions such as hay fever. The slow appearance and persistence, with necrosis of the skin in severe cases, are characteristic. Until recently it was generally considered impossible to transfer this type of sensitization passively by serum from an actively sensitized animal. Transfer by cellular exudates is well established, but all experiments on simple passive transfer with serum were negative or equivocal. Cole and Favour (9), however, have now shown that by concentration and fractionation of large volumes of serum from tuberculous guinea pigs a fraction IV 10 of Cohn allows the passive transfer of delayed reactivity. It appears that the antibody involved has a predilection for cells rather than plasma, so that at any moment there are only small amounts in the circulating plasma although production may be extensive.

Ovalbumin in the guinea pig, as in the rabbit, normally produces only precipitating antibody and classical anaphylactic sensitization, but many years ago Dienes and Schoenheit (10) showed that by inoculating egg albumin into the substance of a tuberculous granuloma delayed tuberculin type sensitization to egg albumin was developed. This indicates a third qualitatively different way in which ovalbumin as an antigen can provoke an immunological response.

Picryl-chloride is a substance of small molecular weight, which when painted on the skin of a guinea pig, gives rise after a week or two to a condition of hypersensitivity with a general resemblance to tuberculin sensitization. Chase (8) by linking picryl to chicken serum produced antigen whose specificity was largely determined by picryl groups. Guinea pigs immunized with the composite antigen produced precipitating antibody and became anaphylactically sensitive, but did not become skin sensitive to picryl. Lymphocytes from the immunized animals were able to transfer some capacity for antibody production, but did not sensitize the recipient. By feeding picryl-chloride to guinea pigs, Battisto and Chase (1) produced a condition of immunological paralysis in that animals so treated (*a*) could not be actively sensitized by normal treatment of the skin and (*b*) resisted anaphylactic sensitization by injection of composite picryl-protein antigens. That this is due to a specific failure of antibody-producing mechanisms to respond was shown by the fact that lymphocytes from "paralyzed" animals were unable to convey passive sensitization, yet the same animals, as recipients, could be passively sensitized by lymphocytes from guinea pigs sensitized in the usual way. If, as we shall contend, this immunological paralysis is a fourth type of specific immunological response, this provides another example of a single antigenic determinant provoking three qualitatively distinct types of response.

Another example of specific immune tolerance is that described by Billingham, Brent, and Medawar (2). This is the tolerance for a heterologous skin graft that is subsequently shown by mice inoculated *in utero* with cells from the donor strain. Two inbred essentially homozygous strains of mice, *A* and *B*, were used. Foetuses in "*A*" mothers about one week before term were inoculated with spleen and kidney cell emulsions from "*B*" mice. When they were some weeks old the white *A* mice inoculated *in utero* accepted a transplant of black *B* skin without reaction. Normal *A* mice would, of course, rapidly develop an immune intolerance and cause the destruction of the foreign graft.

The specific character of the tolerance is shown by the fact that it does not extend to a graft from a third homozygous strain, *C*. That it is due to a modification of antibody-producing cells and not to secondary processes such as absorption of antibody by persisting foreign cells is shown by the fact that a subsequent graft of normal *A* spleen or lymph node will be followed by a progressive breakdown and rejection of the previously tolerated *B* graft (3). There is much to suggest that immune resistance to foreign tissues is a process closely analogous to tuberculin sensitization.

Finally we must mention briefly the condition of immunological inertness that follows the prolonged inoculation of large volumes of foreign protein intravenously in rabbits. Both Hanan and Oyama (15) and Dixon and Maurer (11) agree that when injections of large amounts of bovine serum albumin are commenced soon after birth and continued two or three times weekly for months, no precipitating antibody is produced and even after some months rest to allow the complete elimination of the foreign protein, no response to an injection of bovine serum albumin is elicited. Most but not all of such rabbits will respond normally to another antigen such as ovalbumin.

The Four Basic Types of Response

Many other examples could be given, but these seem to be sufficient to suggest that there are four types of immunological response which may be named.

(1) Classical antibody production.
(2) Hay fever type response.
(3) Tuberculin type response.
(4) Specific immunological tolerance.

It is possible that there are qualitatively intermediate types and there are certainly many instances in which more than one type of response is simultaneously present. There are variations from one species of mammal to another and the different types may not have quite the same interrelations in all species. Enough has been said, however, to indicate the likelihood that there are no fundamental differences in the four species chiefly studied—man, rabbit, guinea pig, and mouse.

To provide a reasonable theoretical frame for this range of qualitatively distinct immunological reactions is the hardest test of any general theory of antibody production.

The self-marker theory of antibody production propounded by Burnet and Fenner (5) was developed from a consideration of the natural forms of prenatally induced tolerance and successfully predicted that experimental methods of producing such tolerance should be found. This theoretical approach has been modified somewhat in a book (4) now in the press and the advances of the past few years make it clear that any such approach will need to be continually restated to keep it in line with developing ideas about the nature of protein synthesis. Equally, however, it may be wise for those whose primary interest is in the nature of protein synthesis to remain aware of facts and ideas emerging from work in immunology and virology.

The main addition to the view presented in 1949 is derived from the ideas that have been developing in the field of adaptive enzymes. Whatever the mechanism of protein synthesis, some means must be available by which significant pattern is transferred from one macromolecule to another. This can be represented by a template *A* which gives rise to the pattern on the protein (antibody, for example) *B*. In line with all who have theorized about

antigen–antibody relationships, we are almost driven to concede that the antigen pattern at one or more removes determines the synthesis of the specific complementary pattern (SCP) on the antibody. Modern work seems to have established convincingly that antigen as such does not persist except under very special circumstances in the immunized animal and that antibody goes on being synthesized long after some antigens have vanished from the body. If this is so, one must postulate some means by which the pattern of a foreign type is stored and kept available (possibly in some different "code") in the cells. This concept of a 'genocopy', i.e. a foreign pattern expressed in the body's own material (ribonucleic acid being the most likely on current views), which can serve as a template for antibody or functionally equivalent protein, is the only novelty in the more recent expression of antibody theory.

From a broadly biological viewpoint, foreign protein or other type of complex macromolecule entering the tissues is undesirable primarily because of its capacity to intrude into and disorganize mechanisms concerned with the synthesis and functioning of the body's own types of such macromolecules. Our picture of the immunological process is based on the contention that its various aspects have evolved to provide ways in which the objectionable interfering components of foreign material can be "naturalized" by being united to protein of body pattern and being dealt with expeditiously as if it were now a damaged fragment of the body's own pattern.

Naturalization at the extracellular level is the function of classical antibody (Type 1), naturalization at the intracellular level is the function manifested as specific acquired tolerance (Type 4). The other two are in a sense intermediate.

On this point of view, the most primitive reaction is Type 4, which we have described as the development of the self-marker mechanism. It is believed that through embryonic life there is a progressive development of specific potentially antigenic patterns and *pari passu* a development, where needed, of 'recognition' mechanisms which allow uncomplicated disposal of effete or misplaced material. One way of picturing the development of a recognition mechanism is as follows. A new macromolecule, either one developed in the body or one introduced from without, entering a cell will cause trouble only if there are portions of its pattern which cannot be broken down by available mechanisms. Such portions may be pictured as reacting (much as penicillin does in experiments with *Bacillus cereus* (20)) with the protein synthetic templates. This term is used simply to indicate the means by which specific functional pattern is conferred upon the protein concerned. It is immaterial whether the pattern is produced at the primary synthesis of the polypeptide chain or as Haurowitz believes, at some later stage in the process. The interference results in the production (*a*) of a protein carrying a SCP for the objectionable pattern of the intruding type of macromolecule and (*b*) of a mechanism by which this new SCP-carrying protein can be produced by the cell concerned and its descendants. In this way a situation is developed by which on entry into the cell the previously new pattern is recognized, i.e. specifically combined with cell protein and so prevented from distorting more

vital aspects of cell activity. The system which includes both the protein with SCP and the mechanism for its continued production can be called a *recognition unit.*

By contrast, the production of classical antibody Type 1 is a function of the developed organism appearing as a rule soon after birth or hatching. Basically the situation is similar to that associated with the production of a recognition unit before birth. A hitherto unknown pattern intrudes into scavenger cells of one sort or another. It is not "recognized" and rendered inert by specific adsorption, and is therefore able to react with protein synthetic mechanisms in essentially similar fashion. The synthetic mechanism, presumably that normally responsible for the production of unpatterned gamma globulin, is induced to produce protein with an appropriate specific complementary pattern and genocopies of the template responsible for conferring that pattern. The new feature is that at some stage in the process the antibody-producing mechanism is transferred as a self-replicating system to cells of the lymphoid series. Here, particularly under the stimulus of a secondary entry of the same antigen, a specialized physiological activity is initiated by which large amounts of the modified gamma globulin are released into the body fluids. Cells that are engaged in active synthesis of such antibody contain large amounts of RNA and have the staining qualities of immature plasma cells (13).

Everything suggests (12) that in the immune animal, antigen and antibody react in the circulation or the tissues and that the product is rapidly removed and both elements of the complex broken down and metabolized. The protection is now at the extracellular level, but it is still designed by coating the objectionable aspects of the material with body protein to allow its uncomplicated disposal in the same fashion as worn out or damaged expendable body cells or components.

Essentially similar processes can be postulated for the Types 2 and 3 responses. The differences amongst the four types can be looked at from two angles, which may be differentiated as physiological and etiological. From the physiological angle, we have differences related to (*a*) the degree to which complete units capable of replicating the new pattern can be transferred to other cells, (*b*) the extent to which the modified protein (antibody) is liberated into the body fluids, and (*c*) the readiness with which the antibody molecules become attached to tissue cells.

From the etiological angle the factors which determine in the adult animal which type of response results, appear to depend (*a*) on the species and genetic constitution of the animal and (*b*) on the type of cells in which the immune response is initiated.

Although classical antibody has been far more thoroughly investigated than either of the other types, it is already becoming clear that neither Type 2 nor Type 3 antibody has the physical qualities of gamma globulin. Loveless and Cann (19) and Cann and Loveless (6) find that the sensitizing antibody (reagin) in hay fever patients is a β-globulin. In a patient who had been

intensively treated and had a high titer of blocking antibody as well as sensitizing antibody, it could be shown that the former was associated with γ-, the latter with β-globulin. Kuhns (16) also found that sensitizing non-precipitating antitoxin migrates more rapidly than standard precipitating antitoxin. It seems highly probable, therefore, that the special character of "hay fever" antibody is that it represents a modified β-globulin and that it is derived from cells in which foreign pattern impinges preferentially on this type of protein synthetic mechanism. It may be that absorption of pollen antigens through nasal mucous membranes brings them predominantly into association with cells of this character. The nature of the antigenic determinants may also be significant, but cellular factors sometimes accentuated by genetic anomalies, seem to be more important.

The finding by Cole and Favour (9) that in tuberculous guinea pigs the plasma protein which is able to convey passive delayed hypersensitivity to tuberculin is in a Cohn fraction IV 10, corresponding neither to β- nor γ- globulin, indicates similarly that Type 3 responses concern a different type of protein synthesis. Purified tuberculin injected alone gives rise to classical type antibody (22) without sensitization. With tubercle bacilli or some of the waxy fractions extracted from tubercle bacilli, a cellular reaction is produced, and it is presumably in some of these cells that the Type 3 process is initiated. The basically similar sensitization of guinea pigs to simple compounds such as picryl, appears to be due to the same type of process. Raffel and Forney (21) showed that typical "contact dermatitis" sensitization to picryl-chloride could be induced in guinea pigs by intraperitoneal injection of the chemical plus purified wax from tubercle bacilli. This must mean that the normal process of sensitization following painting on the skin involves cells of similar type to those which only accumulate in the peritoneum in response to the stimulus of appropriate waxy substances.

"*Immunological Paralysis*"

Of particular interest are the conditions under which antibody production can be specifically "paralyzed" in the adult animal. The best known of these, the effect of large doses of pneumococcal polysaccharide in the mouse, can be dismissed rapidly because recent evidence makes it clear that we are not here concerned with an inhibition of antibody production, but with the persistence of a highly stable antigen, which adsorbs and leads to the destruction of antibody as it is produced (14, 24, 12).

The more interesting examples, which seem to be analogous to the physiological development of specific tolerance in prenatal life, are those in which young rabbits are saturated for long periods with soluble antigen and the inhibitory effect on sensitization of simple chemicals administered to guinea pigs by mouth.

In the first instance we are dealing with a situation where it is obvious that no reasonable degree of antibody production could protect the body cells against the entry of unmodified foreign protein. The production of antibody against the saturating antigen is, therefore, of no significance to the body.

Under such circumstances, it is reasonable to expect that in a rapidly changing population of mesenchymal cells there will tend to be a selective survival of cells, which for any reason have been able to avoid diverting their activity to futile antibody production. Hanan and Oyama's (15) results in which some rabbits developed a *specific* non-reactivity to bovine albumin, while others failed to respond to both bovine serum albumin and ovalbumin, points to the existence of more than one way in which this process of selective adaptation or survival of mesenchymal cells can operate. It seems legitimate to regard specific non-reactivity of this type as equivalent to a reversion to the prenatal form of response.

The phenomenon described by Chase (7) in which guinea pigs are rendered non-reactive to a sensitizing chemical when the agent is administered to the animals by mouth, seems likely to be of special importance, but has not been sufficiently investigated to allow much confidence in interpretation. From the behavior of persons allergic to food proteins, it seems likely that moderate amounts of food proteins in potentially antigenic form are always leaking into the portal circulation. In the great majority of individuals these give no evidence of any immunological activity. The possibility that there is a physiological mechanism by which certain antigens absorbed by the gut can render the body generally immunologically non-reactive to them may have to be considered, although so far the only positive evidence is that provided by Chase. The fact that there is a failure to react to the standard sensitizing treatment on any portion of the skin must mean that cells remote from those in immediate relation to the intestine must be equally influenced. The possibilities are (*a*) that in the course of entry from the gut the determinant unites to a body protein—the complex passes widely in the blood and wherever it enters a RE cell induces, because of its intrinsic character, the condition of specific immunological tolerance; (*b*) that in some particular set of cells, perhaps in mesenteric lymph nodes, a response of the recognition unit type is set up, which, in a way somewhat analogous to what we are forced to postulate for the production of classical antibody, can be transferred to reticuloendothelial cells throughout the body. The dilemma is virtually the same as still remains unsolved for standard antibody production in which the process is initiated in the macrophage cells, but antibody production appears limited to the lymphoid series. There is still no decisive evidence as to whether the stem cells of the lymphocytic series receive from the macrophages broken down antigen or the prototype of an antibody-producing mechanism.

Conclusion

Everything in this discussion is tentative. It is almost in the nature of things that generalizations that are not subject to test by the demonstration of a precise quantitative correspondence of relevant observations, must always be subject to modification with each extension of the experimental or observational findings. This does not mean that such provisional generalizations are

useless. They can at least serve as guides to the directions in which experimental work is likely to be fruitful. The qualitative differences in the type of immune response with which we are concerned are clearly not covered by the standard Haurowitz–Mudd theory of antibody production. Only the outlines of an alternative to this have been sketched. Further development is likely to come from the sort of detailed study of Types 2, 3, and 4 responses that classical antibody production (Type 1) has received. Perhaps of all the fields available for study, the problems of acquired specific tolerance, both pre- and postnatal, offer the best promise of a fruitful harvest of new ideas.

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DYSENTERY BACILLI—SHIGELLA

A BRIEF HISTORICAL REVIEW[1]

By H. J. Bensted

Abstract

This is a brief history of the bacteriology of bacillary dysentery from the time of its differentiation from the amoebic disease up to the present time. It is meant to be a story of events rather than a critical essay. A certain amount of background has been introduced to indicate the problems of identification and classification of the various bacterial types of the disease. Inevitably more space has been given to the mannite-fermenting organisms since the problem of their antigenic structure and interrelationship took so many years to solve. The events leading up to the present serological classification of the dysentery bacilli are set out and certain other problems e.g., those related to the carriage of *Shigella* antigens by other members of the Enterobacteriaceae are discussed.

The first step in the differentiation of amoebic from bacillary dysentery may be said to have been taken by Losch (40) who, in 1875, described the amoeba of dysentery and experimentally infected a dog with an amoebic exudate, but apparently he did not at that time claim that the amoebae were the cause of the disease. There was a long interval before pathologists were able to show that amoebic and bacillary dysentery, although they could occur together in the same individual, were separate diseases caused by entirely different agents. The crowning efforts were in large part due to the painstaking work in 1891 by Councilman and Lafleur (20) and in 1893 by Kruse and Pasquale (36). But the infective agent of bacillary dysentery had yet to be defined. It is true that in 1888 Chantemesse and Widal (15) had described an organism which they claimed was responsible for epidemic dysentery, but the description was very inadequate and although in 1903 it was shown by Vaillard and Dopter (56) that the cultures of Chantemesse and Widal which, by a stroke of fortune had been preserved, were in fact identical with the true *Bacillus dysenteriae* discovered by Shiga (48, 49) in 1898. Most authorities give the credit to Shiga but the French have always regarded Chantemesse and Widal, in spite of the inadequacy of their original description of the organism, as the true discoverers of *B. dysenteriae*. While investigating an epidemic of dysentery in Japan, Shiga (48) cultured from the faeces of one of the patients an organism which was agglutinated by the serum of the same patient whereas the ordinary *Bacillus coli* on the plate culture were not. Moreover this serum agglutinated similar organisms cultivated from the stools of a number of other dysentery patients where they were often present in pure culture. Serum from healthy individuals or those suffering from other diseases showed no such reaction. In 1900, Flexner (29) and Strong and Musgrave (51) working in the Philippine Islands all reported similar findings and in the same year Kruse (34) described an organism which he had isolated from nearly every case examined in an outbreak in Germany. Kruse, however, found his organism to be non-motile but because Shiga, Flexner, and the others had stated that their organism was sluggishly motile, he claimed that his was different and

[1]*Manuscript received October 19, 1955.*
Contribution from the Central Public Health Laboratory, London, United Kingdom.

consequently a new discovery although the two appeared to be serologically identical. There was considerable controversy at the time and it was not until a special board was able to convince both sides that the two organisms were one and the same and non-motile that peace reigned. The organism henceforth was generally known as the Shiga–Kruse bacillus.

In the following year Kruse (35), investigating an outbreak of Asylum dysentery in Germany, isolated another non-motile organism superficially resembling *B. dysenteriae* but which was serologically distinct. Because the disease was clinically less severe he called the organism *Bacillus pseudodysenteriae.*

The cultures isolated by Flexner and by Strong and Musgrave were received in Germany and Martini and Lentz (43) proceeded to undertake an extensive serological examination of these together with strains from other sources. They found that Shiga's, Kruse's, and one of Flexner's strains were identical in every way, but that another of Flexner's cultures and that from Strong were the same as Kruse's pseudodysentery organism. Moreover they found that the latter group fermented glucose and mannite whereas those agreeing with the original Shiga organism fermented glucose only. The mannite fermenters (Kruse's pseudodysentery bacilli), in spite of certain minor biochemical differences, appeared to be serologically related but not identical. The non-mannite fermenters however were all identical both serologically and biochemically and agreed in every way with the Shiga–Kruse *B. dysenteriae* so that the two types could be clearly differentiated.

In 1903, Hiss and Russell (32) and Hiss (31) suggested a method of classification which was known by their name but it was essentially the same as that put forward the previous year by Lentz (39) and depended on the fermentation reactions obtained with five sugars, lactose, glucose, mannite, maltose, and saccharose, to differentiate the Shiga–Kruse organism from the other type. It may be recalled that Lentz (39) did not favor identification by biochemical reactions owing to the irregular results and the necessary delay before the results were available. He much preferred serological diagnosis which he claimed gave exact results in a matter of minutes.

Up to this time there was no record of British bacteriologists investigating dysentery bacilli but in 1903 and in the following year Charles Todd (54, 55), then working at the Lister Institute, published two papers on the subject. They were mainly concerned with the Shiga toxin and antitoxin. The early workers had all appreciated the toxic nature of *B. dysenteriae* cultures and the credit for the discovery of the toxin is usually given to Conradi (19) but Todd himself made important observations. He showed that Shiga cultures produced a soluble specific toxin and that the homologous antiserum contained not only antibacterial substances such as agglutinins, but also protective or neutralizing substances specific against the toxin itself—antitoxin. He also showed that all the non-mannite-fermenting cultures of *B. dysenteriae* he tested produced this toxin whereas not one of the mannite-fermenting pseudodysentery bacilli showed any evidence of this property.

Early in 1904 Eyre (27) had isolated a number of cultures of non-mannite-fermenting *B. dysenteriae* from severe cases of Asylum dysentery (the mannite-fermenting strain was however more common in the less severe cases as Kruse, and also subsequent workers, e.g. Aveline, Boycott, and Macdonald (4) had found). Todd in his experiments used the classical Shiga strain, the Kruse strain, and three of Eyre's strains for the non-mannite fermenters and a number of mannite-fermenting strains from different sources, and found that the former group were all identical in their biochemical, serological, and toxicological reactions with the original *B. dysenteriae* of Shiga, and thus differed essentially, in each of these tests, from the mannite fermenters which Todd called *B. dysenteriae* Flexner.

Todd's paper is also of special interest in that there is clear evidence of the availability at that time of classical strains of dysentery bacilli from other laboratories. Todd obtained, or the Lister Institute possessed, subcultures of the original Shiga and Kruse strains and also mannite-fermenting strains from Flexner, Duval, and others, some of which were obtained from Dr. Kral's laboratory in Prague—then internationally known for its collection of bacterial cultures.

Since that date there appears to be no record of the receipt of dysentery cultures from Kral's laboratory or other foreign source until after World War I.

Castellani (13) writing from Ceylon in 1904, reported the isolation from dysenteric stools of non-lactose, mannite-fermenting, indole positive non-motile organisms, identical serologically with Kruse's pseudodysentery bacilli. He expressed disapproval of Kruse's nomenclature (35) and suggested that the condition would be better named paradysentery and the organism *Bacillus paradysentericus*. It would appear that Castellani was the first to use this name although Collins (18) is usually given the credit for '*B. paradysenteriae*' which name she used in 1905 and was followed in this by many American bacteriologists from that time.

In 1907 Kruse *et al.* (37) published the results of an extensive biochemical and serological investigation of his pseudodysentery bacilli by which means he separated the group into a number of related but serologically not identical races (A to H). One of these, however—Type E— was a late-lactose fermenter and was serologically distinct from the others. Organisms having the same characters had been isolated by Duval (22) and it would seem that Castellani's *Bacillus ceylonensis* A (14) was also identical. (In acknowledgment of Sonne's (50) intense studies on bacillary dysentery due to this organism it eventually received the name *B. dysenteriae* Sonne.)

Kruse appears to have been the first worker to make extensive use of serological methods, including absorption techniques, in studying the mannite-fermenting strains. The published account of his work was fully appreciated in Germany and certain other countries but in English speaking countries it appears to have been unknown or ignored except by a very few bacteriologists.

There was little exchange of type-cultures between workers of other nations and relatively few British bacteriologists employed the techniques used in Germany for the exact identification of these bacteria.

At the outbreak of the 1914–18 war, there was little evidence of any detailed knowledge in Great Britain or America of Kruse's work, nor were authentic cultures of his different races to be found in British laboratories. The high incidence of dysentery in all theaters of the war led to an intensive study of the bacteriology of the disease by a number of British workers. The main division between the Shiga organism and the Flexner group was, of course, widely appreciated and it was recognized that the differentiation between the members of the latter group constituted the major problem. To this end numbers of strains were collected from various theaters of the war for examination. The two major investigations were undertaken by E. G. D. Murray (44), working at the R.A.M. College, Millbank; and Andrewes and Inman (1) at St. Bartholomew's Hospital. L. Dudgeon (21), Gettings (30), and others also made important observations. The results of the very careful investigations of these groups of workers were in close agreement with each other and with the earlier work of Kruse. The published accounts of the work of Murray and of Andrewes and Inman were acclaimed by the English speaking world and at once took an honorable place in the classical literature on the bacteriology of dysentery. Like Kruse, they had been able to show that the mannite-fermenting dysentery bacilli could be divided into a number of related races in which the serological differentiation was not sufficiently distinct to enable specific antigenic types to be defined. It would seem that the early workers had in mind the possibility of classifying this group of organisms by demonstrating specific type antigens, but Murray (44) and Andrewes and Inman (1) concluded from their investigations that each race contained four antigens and according to their quantitative representation so a type could be defined. Symbols V, W, etc., were given to these antigen components to prevent confusion with Kruse's (37) symbols, which began at the other end of the alphabet, and Andrewes' type V for example was defined as one containing a very large amount of the V antigen and small amounts each of W, X, and Z. The Y strains were said to contain more or less equal representation of all four antigens.

This British work was essentially a serological classification based on quantitative analysis and both Murray (44) and Andrewes and Inman (1) made it clear that they did not favor any attempt to employ detailed biochemical tests for classification purposes beyond the main division in accordance with the reaction with mannite. It is true that their results did not carry fundamental knowledge of the antigenic structure of the Flexner group very much further than Kruse had done more than 10 years previously, but these the first major publications on dysentery bacilli in English did offer an explanation of the interrelationship within the group and it had a great influence on the work of other bacteriologists over the next 10 to 15 years.

Schmitz (47) had isolated in 1917 from typical clinical cases of dysentery in Roumania a non-mannite indole-positive organism that differed serologically from the Shiga–Kruse bacillus (the *B. ambiguus* of Andrewes (1) is identical). There were thus three types (Shiga–Kruse, Schmitz, and Sonne) with relatively simple antigenic structures which could be readily defined serologically and the large important group of so-called pseudodysentery, paradysentery, or Flexner organisms with an incompletely understood antigenic make-up which still defied proper classification.

Martini and Lentz (43) and Hiss (31) had divided *Shigella* into two main groups and Kruse's extensive investigation in 1907 had, with his serologically distinctive group E, added a third member. Murray (44) included *B. dysenteriae* (Schmitz) in the scheme to make a total of four members: *B. dysenteriae* Shiga, Schmitz, Flexner, Kruse E (Sonne).

Modifications of this scheme were put forward by Thjøtta (53), Aoki (3), Sartorius and Reploh (46), Bojlen (6) etc., but they were not fundamentally different from Murray's scheme. Thus in spite of the intensive work of a long line of eminent bacteriologists it had only been possible to divide the Flexner group into a number of closely interrelated types without being able to show, with any convincing evidence, the nature of this relationship. The mosaic hypothesis put forward by Murray (44) and Andrewes and Inman (1) was attractive and was accepted by the majority of English speaking bacteriologists but it was not completely satisfying to many. It was obvious that there were still gaps in our knowledge of the antigenic structure of the group and that a more lasting classification would be possible only when more exact information was available.

Hitherto the great majority of the strains investigated had been isolated from patients involved in an outbreak of dysentery or from soldiers during the first World War in which conditions might be regarded as similar.

In 1929 J. S. K. Boyd (7), who was then working in Bangalore, commenced his investigation of the mannite-fermenting dysentery bacilli with the primary object of defining more clearly the so-called inagglutinable Flexners. These were a group of organisms that had the general characters of the Flexner bacilli but which were not agglutinated by any of the sera made from the Andrewes and Inman types. (Little had been published about these inagglutinable types but they were well recognized by working bacteriologists in the Near East as organisms of importance, and following Dudgeon (21) the present author always employed a polyvalent serum prepared from two or more local serotypes of 'inagglutinable Flexners'.) Boyd's project led eventually to the collection of more than 5000 cultures from genuine cases, mostly unassociated, of bacillary dysentery from widely scattered stations all over India. These were compared with a number of classical strains from a variety of sources.

The results of the first studies (7), which were published in 1931, contained the key to Boyd's later work. One of the strains of Flexner-like organisms appeared on first isolation to be a distinct serological type and to fall into this so-called 'inagglutinable Flexner group'. However, on laboratory media

variants developed and some cultures of the strain which originally reacted only with the specific homologous antiserum and not with any of the Flexner group, were now agglutinated equally well by unabsorbed sera made from any of Andrewes' types V to Z and not at all or only very slightly by the original homologous serum. By examining a number of similar strains he was able to show that this new type (No. 103 in his series) possessed an antigen complex consisting of a type specific substance unrelated to any of Andrewes and Inman's types (V, W, or Z) and a group antigen, components of which were common to the other members. In addition he was able to show that degradation or antigen variation could proceed either slowly or suddenly, resulting in some cases in complete loss of the specific substance almost overnight. In this and his later papers (8, 9, 10, 11) Boyd was able to show that what was true with regard to this type '103' and two other new types he isolated (Boyd's No. 88 and 119) was also true of the classical types V, W, and Z and that the so-called X and Y types were in fact not valid types but pure group variants with complete loss of their specific substance. In addition he found some new serological types with a simpler antigenic structure, each with a well-defined specific substance but with no evidence whatever of any common group antigen.

Here then was a more satisfying explanation of the antigenic make-up of the Flexner group backed by sufficient evidence to warrant a comprehensive classification of the mannite-fermenting dysentery bacilli thus:

(*a*) Those possessing an identifiable specific antigen together with a group antigen, components of which were common to the other members.

Flexner 1–6.—These comprised the three classical types of Andrewes and Inman (V, W, and Z) and three new types 103, P119, and 88. (P119 was identical with IV of Aoki (3) and G of Sartorius (46), and 88 was serologically identical with the type Newcastle described by Clayton and Warren (17)).

(*b*) Those possessing a specific antigen only which was unrelated to that carried by any other dysentery organism.

Boyd 1–6.—These were all new types hitherto undescribed although it is probable that one or more of these types were encountered by Dudgeon and others (21) and included in his 'inagglutinable Flexner group' but these organisms were not fully investigated by previous workers.

Although there was some minor disagreement with regard to the exact structure of the group-complex and a certain amount of resistance from those who still favored the mosaic hypothesis, Boyd's work was not seriously challenged but was in the main fully confirmed and accepted throughout the world. (Wheeler (58, 59), Weil, Black, and Farsetta (57), Fergusson *et al.* (28), Carlquist (12), and Madsen (42)).

Several of these authors have modified Boyd's classifications but none has been essentially different from that proposed by him nor has any subsequent work done more than confirmed the correctness of the principles established by him. The additional subtypes that are now recognized were to be expected once these principles were accepted.

Some additions to the Boyd group were thought to be inevitable by the present author (5) but the fact that no more than five new types, all of which may be regarded as relatively uncommon, have been added in the last 15 years, is sufficient evidence of the thoroughness of Boyd's original survey.

Boyd's work was by far the most important of all modern investigations on the antigenic structure of the Flexner group of organisms but in the year following his first paper on the subject Clauberg (16) published a report of his absorption experiments with a number of Kruse's races by which he demonstrated the presence of specific and group antigens. The work however, was incomplete and the results not quite in accordance with those of Boyd and those who were able to confirm his findings. Kauffmann (33) also had made observations which pointed to a similar conclusion.

The Large–Sachs Group

Large and Sankaran (38), working in Quetta in 1934, isolated from dysentery patients non-mannite-fermenting organisms which were serologically distinct from the Shiga–Kruse or Schmitz organisms. Their work was extended by Sachs (45) and a number of different serotypes were identified. There seems little doubt that the non-indole non-mannite-fermenting dysentery bacilli described in 1919 by Dudgeon (21) belonged to this group. MacLennan (41) working in Egypt during the late war examined a considerable series of non-mannite-fermenting dysentery organisms and readily identified most of them with one or other of the strains described by Large and Sankaran and Sachs from India. Wheeler and Stuart (60) in their investigation of dysentery organisms of the non-mannite-fermenting group in U.S. soldiers were able to confirm the findings of the previous authors. Wheeler and Stuart agreed that the Large–Sachs Q-group could be classified as dysentery organisms but did not go as far as admitting to the membership of the *Shigella dysenteriae*. Eventually five distinct Large–Sachs serotypes, Q771, Q1167, Q1030, Q454, Q902 were proposed for acceptance as valid dysentery types.

At this stage it is perhaps suitable to record that in 1950 at the Congress of the International Association of Microbiologists in Rio de Janeiro the Shigella Commission made certain recommendations (23) based on the scheme of Ewing (24) with regard to the classification of the dysentery bacilli. These may be very briefly summarized as follows:

(1) It was recommended that the name *Shigella* be adopted as the generic name of the group of intestinal bacteria commonly known as the dysentery bacilli or *B. dysenteriae*.

(2) That the genus be defined as consisting of Gram-negative, non-motile, non-sporing rods corresponding to *Shigella dysenteriae* (Shiga's bacillus) in morphology and staining properties. All members ferment glucose, some ferment mannitol and, with a few exceptions, all are nonproductive of gas from fermentable substances. They do not acidify salicin or adonitol, nor grow on

Simmons' citrate agar, or hydrolyze urea, nor liquefy gelatin, nor form acetyl-methyl-carbinol. Lactose is fermented by *Shigella sonnei* but only after prolonged incubation. All members of the genus have an antigenic structure by which they can be recognized.

SHIGELLA GROUP

Subgroup A. (All non-lactose, non-mannite fermenters)

Shigella dysenteriae. Valid types No. 1 to 7.

Shiga's bacillus to occupy the first place, Schmitz the second, and the Large–Sachs types taking the other places. Any additional types that may be accepted in the future would take their place in order.

Subgroup B. (Non-lactose, mannite fermenters. Each member possessing a distinct type antigen together with a complex group antigen, components of which are found in other types within the subgroup).

Shigella flexneri. Valid types No. 1 to 6 with subtypes labelled *a* or *g*. Pure group variants which represent total loss of specific type antigen to be known as X and Y respectively.

Subgroup C. (Non-lactose, mannite fermenters, each member possessing a distinct type antigen but no characteristic group complex).

Shigella boydii. Valid types No. 1–11.

Any new valid types that may be accepted would take their place in order.

Subgroup D. (Slow lactose and mannite-fermenting bacilli.)

Shigella sonnei.

It was further recommended that all members of the *alkalescens–dispar* group, together with a number of less important types, be removed from the *Shigella* group. The biochemical characters of the *alkalescens–dispar* group showed them to be close to the *Escherichia* group.

These recommendations were put forward on a majority vote (23). The difference of opinion among the members of the Commission arose over the nomenclature of Subgroup A, which was to include as equal members all the non-mannite fermenters under the binomial *Shigella dysenteriae* with differentiating numbers only. The name *Bacillus dysenteriae* was applied as long ago as 1898 by Shiga himself (49) to a specific bacterial type with distinct and peculiar properties and the dissenting members of the Commission felt that it was inconsistent to apply also this special name, with all its historical associations, to other and less important organisms. The position regarding the names to be applied to the other subgroups was entirely different since their species names were all derived from the surnames of bacteriologists whose work was closely associated with the organisms concerned. The objection, which was overruled, it is true, was raised by two medical bacteriologists who had intimate knowledge of Shiga dysentery and the Shiga bacillus for well over 30 years and who could not regard the Schmitz and the Large–Sachs organisms as the equal of the Shiga bacillus.

A possible solution to the difficulty would be to discard the species name and to retain the symbols of subgroup headings A, B, C, and D and to use arabic numerals to indicate the specific serotypes.

It will be recalled that ever since Andrewes' (1) time *Bacillus alkalescens* and *Bacillus dispar* have been looked upon by bacteriologists with some suspicion and doubt as to their etiological claims to be regarded as dysentery bacilli; now a good reason has been found for excluding them from *Shigella* on biochemical grounds (52). It is satisfying that the definition enables *S. sonnei* to remain in the group; the medical bacteriologist accepts this with relief for after more than 50 years as a dysentery bacillus many would be disconcerted to see the Sonne organism relegated to *Escherichia*. The definition also neatly overcomes the difficulties that could arise over the very wide variation in the biochemical reactions of *Shigella flexneri* 6 (Newcastle–Manchester–Boyd 88) and a few others (26) and it readily disposes of those organisms showing various degrees of antigenic relationship to *Shigella* but which are quite unrelated in their cultural and biochemical reactions. It has been appreciated for many years by medical bacteriologists that some non-*Shigella* organisms have serological relationship with dysentery organisms, but it was the general practice to discard them without detailed investigation. However, with the growing knowledge of the antigenic structure of the *Escherichia* and allied groups which we owe to Kauffmann (33) and his colleagues and the complete change of approach to intestinal bacteriology, increasing numbers of cultures showing these relationships are being identified. Ewing (25) investigated in detail the serological and biochemical reactions of a considerable number of these cultures. In many cases the *Shigella* relationship was distant or of a minor nature and the biochemical characters unmistakably different. Yet there were others where the antigenic make-up appeared to be identical and the preliminary biochemical reactions not completely dissimilar. The present writer (unpublished) has occasionally isolated from stools of convalescent dysentery patients non-lactose fermenting non-dysentery organisms with minor serological relationships to the dysentery organism isolated during the acute disease. And Ewing (25) quotes more than one occasion when *Escherichia coli* C27—a motile slow lactose-fermenting anaerogenic strain which is related serologically to *S. sonnei*—has been isolated side-by-side with true *S. sonnei* from the same stool. This worker has also found that antigens of almost every *Shigella* type and subtype could be demonstrated in *E. coli* and allied groups.

The present scheme for the classifying of the *Shigella* organisms follows the broad methods suggested soon after the turn of the century. The incomplete knowledge of the antigenic structure of the lactose-negative mannite-fermenting dysentery bacilli did not affect the main classification of the whole group. When Boyd in 1931 (7) discovered the key to their antigenic make-up he exploited it to the full, but there was no disruption of the general scheme; each type fell into its place in two subgroups according to whether or not it possessed the characteristic group antigen in addition to its type specific antigen.

It will be recalled that Lentz (39), Murray (44), and Andrewes and Inman (1) all relied on a short range only of carbohydrate media for their preliminary classification and that practically all bacteriologists working with the *Shigella* group from early times down to the present day have relied almost entirely on serological identification for classification purposes.

This system worked well enough so long as bacteriologists were concerned solely with organisms causing clinical dysentery and Boyd in collecting material for his investigation was careful to ensure that his cultures originated from cases of true bacillary dysentery. But with the postwar intensive bacteriological studies concerned with the control of intestinal infections and the increased knowledge of the antigenic structure of the so-called non-pathogenic Enterobacteriaceae more and more evidence of the antigenic interrelationship between the groups of this family is accumulating. These groups are biochemically defined so that additional biochemical tests must of necessity be introduced in the routine identification methods. They are of special importance in the case of any new *Shigella* serotype and also with recognized serotypes isolated from apparently normal stools. It is at present difficult to see what will be the outcome of this awakened interest in detailed biochemical tests for the *Shigella* organisms. But it must be remembered that the present compromise system is essentially practical and holds together in one group all the dysentery producing bacteria. This may not go unchallenged in the future and a recent annotation in the Lancet (2) suggests that there are signs of dissatisfaction among some pure taxonomists with a definition that allows the present system of classification.

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MILESTONES IN THE HISTORY OF THE MENINGOCOCCUS[1]

By Sara E. Branham

Abstract

This paper cites the most significant findings in the development of present day knowledge of the meningococcus (*Neisseria meningitidis*). It is pointed out that advances have occurred stepwise, each step involving approximately a decade. These decades are discussed in turn: the period of classification, the development of serum therapy, epidemiology and biology, chemotherapy and resistance, and finally transduction and genetic recombination. Future trends in research on the meningococcus are pointed out.

Present knowledge of *Neisseria meningitidis*, accumulated since Weichselbaum first found it in 1887 (84), is not the result of a steady growth. This fact is at least partly due to the epidemiological habits of the organism. Acquisition of information has come chiefly during epidemic times. Some years have been rich indeed and other extended periods contributed little.

This paper is not a review of the voluminous literature that has accumulated concerning the meningococcus, but it includes only events which were of special significance in influencing the trend of investigation in the field. These developments did not actually form a chain for they were not all linked together; rather, they were sign posts that pointed the way, or milestones that marked points of progress. In a general way the acquisition of information has fallen into decades, owing, in all probability, to the cycles in which epidemic meningitis occurs, usually about 8 to 12 years apart (38).

The Period of Classification

In a special sense this was from 1909 through 1918. It is true that the classifications in common use in recent times, and employed throughout this brief review, were developed much later. However, during these years classification was the principal interest and was the chief contribution of that decade. The period began in 1909 when Dopter (24) reported that meningococci were not all alike serologically. But the most intensive work was done during World War I which brought widespread epidemics of meningococcus meningitis among the armed forces and civilians of all nations participating. A tremendous amount of work was done in several countries, especially during 1915–1918, during which time at least six different classifications were proposed (35, 80, 34, 62). The I, II, III, IV type classification of Gordon and Murray (34) was adopted by practically all English speaking countries, and the French A, B, C, D classification (62) was used extensively in Continental Europe.

[1]*Manuscript received September 6, 1955.*
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Many years later revisions were made in the commonly used nomenclature, (22, 13, 17), and in 1950 the Subcommittee on *Neisseria*, of the Nomenclature Committee of the International Association of Microbiologists, proposed a classification of the meningococcus (82) based on the International Code of Nomenclature (39).

A discussion of all of the reported classifications of the meningococcus, as well as a table indicating the relation of these to each other is given by Branham in a recent review (11). A simplified version of this table seems necessary for the understanding of this present discussion, and is included here as Table I. The classification recommended by the Committee in 1950 will be used in this paper. After this new classification was recommended it was found that Murray had proposed a similar one in 1929 (61), in his monograph "The Meningococcus".

TABLE I

RELATION AMONG MOST COMMONLY USED CLASSIFICATIONS OF THE MENINGOCOCCUS

Gordon and Murray	1915	I	II	III	IV		
Nicolle, Debains, Jouan	1918	A	B	—	—	C	D
Common use since	1940	I	II	I	IV	II_a	
Recommended by Committee	1950	A	B	A	D	C	

Development of Serum Therapy—1906–1913

Introduction of therapeutic serum for meningococcus meningitis by Jochman (40) and its establishment on an accepted footing by Flexner (30) was an important step for that era. Therapeutic serum had its greatest usefulness during the period of World War I. The beginning of this period actually antedated that of classification, and was undoubtedly a stimulus for serologic studies. Conversely, the serologic studies were responsible for the development of polyvalency in serum.

Epidemiology and Biology of the Meningococcus—1928–1940

During the decade following World War I (1918–1928) this field lay fallow. Strains of meningococci, considered representative of the various serological groups, were maintained, and therapeutic sera made and tested for agglutination titer.

The tremendous eastward-moving epidemic wave, apparently beginning in China, that reached the West Coast of the United States by way of the Philippines in 1928, marked the beginning of a new era. It was quickly realized that the valuable studies in classification made during the World War I years were only a foundation, or rather, a framework, on which knowledge of the meningococcus itself was to be superimposed.

The decade that followed was rich and fruitful. Knowledge developed rapidly along two lines which were so closely integrated that they cannot be adequately discussed apart: epidemiology and biology of the meningococcus.

Epidemiology

As the wave of meningococcus infection moved across the United States and Canada hundreds of strains of meningococci were recovered and typed by many people in many places – in hospitals, institutes of various kinds, public health laboratories, civilian communities, and military installations. The majority of these were from cases of meningitis, but a number were from carriers. From these serological studies the following indubitable facts emerged:

(1) The types I and III of Gordon and Murray were so closely related that it was impractical to attempt routinely the agglutination absorption tests necessary to separate them. Even their specific capsular polysaccharides were shown to be identical (8, 12, 16, 64, 71, 74). They were thereafter designated together as Group I (new designation A).

(2) As many as 95% of the strains from cases of meningococcus infection were found to be of serological Group A (formerly I) during the peak of the epidemic. The proportion of Group B (formerly II) strains increased only as the epidemic began to wane. In subsequent epidemics this same distribution has been found, and a search through older literature gives considerable evidence that a similar situation was noted during World War I by some investigators (11). Hence Group A (old Group I) has become known as the epidemic group. Outbreaks due to other groups do occur also, but they are apt to be limited.

(3) During epidemics meningococci of Group A (I) are found in carriers frequently. However it is usually Group B (II) that is found in chronic carriers, and other carriers during interepidemic times. Such carriers are doubtless responsible for sporadic cases which are usually due to B (II) or C (II α) (12).

Laybourn (42) demonstrated the fallacy of the old theory that an epidemic occurred when the meningococcus carrier rate reached 20% of the population. He showed clearly that it is not the number of meningococci or of carriers that creates a menace, but the nature and virulence of the organisms carried. This observation has been repeatedly confirmed, i.e. by Rake (72), Maxcy (50), Miller (59), and Aycock (3).

Biology

Petrie (66) found that "fresh" meningococcus cultures of Group A produced halos of precipitate on homologous serum agar, whereas old stock cultures of that serological group did not. Maegraith (49) showed that this was a manifestation of "smooth" and "rough" nature respectively. Rake (71) found striking antigenic differences between these variants. The use of "smooth" cultures for preparation of agglutinating and precipitating sera resulted in high specificity and titer. Suspensions were agglutinated specifically after two hours incubation at 37° C. instead of at 55° C. overnight, as had been customary. These findings were of immediate practical value. For many years therapeutic sera had been prepared with old stock cultures, many of

which were rough. Such sera were titrated by an agglutination test held overnight at 55° C., a temperature which was now shown to agglutinate non-specific as well as specific antigens. Improvement in the quality of therapeutic serum resulted promptly.

In 1933 Rake and Scherp (74) described the type specific substance of Group A meningococcus. They isolated it from the capsular substance of the cells and found it to be a sodium salt of a polysaccharide acid. This polysaccharide is associated with the specificity, the smoothness, the antigenicity, and the virulence of these meningococci. No comparable polysaccharide has been isolated, thus far, from other serological groups, although the behavior of C (old II α) strains indicates that such a substance is present.

In 1931 Rake (70) had pointed out the presence of capsules on freshly isolated strains of meningoccoci. Capsule swelling, using a specific serum rich in precipitins, was suggested by Clapp (21) as a method of typing. This was reported in more detail by Beckler (5) and by Milner and Shaffer (60). This very practical diagnostic aid has made possible direct typing from spinal fluids within a few minutes time. Capsule swelling can be done with Group C meningococci as well as with Group A though the specific capsular substance of Group C has not been studied chemically. No capsule has been demonstrated on Group B organisms though production of "halos" of precipitates by smooth virulent strains with homologous sera indicates specific antigens. There has been no opportunity to make such a study of Group D because of its rarity. Usually capsule swelling tests are performed with antisera prepared in rabbits. Milner and Shaffer used antisera prepared in chickens by the method described by Phair, Smith, and Root (68). Such chicken sera were found very useful in field laboratories during World War II for typing meningococci by the quick agglutination technique described by Noble (63).

In 1933 Miller (51) described meningococcus infection in mice following the injection of a few cells of smooth fresh strains suspended in hog gastric mucin. Until this time such infections could be produced only by giving massive doses of highly virulent cultures, a procedure that resulted in an overwhelming intoxication which often masked any true infection which occurred. Miller's report opened a new door for the study of the meningococcus. By use of this technique, both Rake (73) and Miller and his co-workers, (59) were able to demonstrate degrees of virulence of carrier strains, a matter of considerable epidemiological significance. Its use made possible the *in vivo* testing of the therapeutic activity of drugs and antibiotics. Titration of therapeutic potency of antimeningococcus serum, formerly strictly a test tube affair, could now be done on a protection basis. The working out of a standard method of performing this titration by Branham and Pittman, combined with use of a preliminary test for halo production on agar plates (14), resulted in further improvement of therapeutic antimeningococcic serum. Only sera containing specific precipitation antibodies for Groups A, B, and C gave protection to mice against meningococci of the homologous group.

During this decade, also, came the development of the freeze-dry process designated by Flosdorf and Mudd as "lyophilization" (32). Now smooth, virulent, encapsulated meningococci could be preserved as such, and kept unchanged for long periods of time. The benefit given to work with the meningococcus by this method is inestimable.

Chemotherapy and Resistance—1940–1950

Studies of the effect of sulphonamides on meningococcus infections began in 1936 and 1937 with Buttle, Gray, and Stephenson (19) and with Proom (69) who described the protection of mice against meningococcus infection with sulphanilamide. Also in 1937 Branham and Rosenthal (15) reported the synergistic action of sulphanilamide, Prontosil, and Prontosil Soluble with therapeutic serum in protecting mice against meningococcus infection and during the same year this was confirmed by Brown (18). During this year Schwentker (78, 79) began treatment of human patients with sulphanilamide alone, and in 1939 Long (47) reported better results in patients with sulphapyridine.

Just at the time when superiority of the monovalent rabbit serum (10) over the older polyvalent horse serum was beginning to be known, it became evident that no serum at all was actually necessary, so that its use in meningococcus infections was soon completely discontinued.

No situation is without its disadvantages and this good fortune with sulphonamides in meningococcus infections was accompanied by one less fortunate corollary: when biological manufacturers discontinued making therapeutic antimeningococcic serum they also stopped preparing diagnostic serum, a circumstance which has resulted in great inconvenience for many concerned.

During the decade following the therapeutic use of these earlier sulphonamides many newer ones were introduced. These have had advantages such as greater solubility, increased effectiveness so that smaller doses could be given, and absence of some undesirable side reactions. All meningococci are not equally susceptible to sulphonamides. This variation will be discussed later on.

Meningococci are susceptible to penicillin. Fleming's discovery of penicillin (29) and its purification by Florey and Chain (31) opened another new gate to a fruitful new field in microbiology.

Meningococci are also susceptible to the so-called broad spectrum antibiotics: chloramphenicol (4, 27), aureomycin (26), and terramycin (28), which were developed in rapid succession. Quite different in effect from the four above mentioned antibiotics is the dramatic and extremely valuable streptomycin (76) which is contraindicated in meningococcus infections because of the rapid changes in response of meningococci to this agent (54, 55).

Antibiotics of bacterial origin, such as bacitracin (41), polymyxin (6, 81), and tyrothricin, are little used in treating meningococcus infections; relatively few strains are sensitive to them and their toxicity is a contraindication.

However, tyrothricin (25) made from *Bacillus brevis* does have a special use in work with the meningococcus, especially in carrier surveys, because of the relative insensitivity of these microorganisms to this agent. When added to blood agar plates in suitable dilutions it inhibits the growth of such common nasopharyngeal bacteria as staphylococci, streptococci, and pneumococci, allowing relatively undisputed territory for the growth of all species of *Neisseria*, as well as *Haemophilus* and other insusceptible organisms. Schoenbach (77) reported this technique for isolating *Haemophilus influenzae* but did not consider it successful for the meningococcus. However, during a carrier survey made in 1942, the present author used it successfully for isolation of meningococci from nasopharyngeal cultures when the final dilution of tyrothricin in the blood agar medium was 1–10,000 to 1–100,000.

In spite of the widespread use of penicillin and the "broad spectrum" antibiotics, sulphonamides continue to be the agents preferred by many clinicians for treatment of meningococcus infections. Sulphadiazine is the drug of choice, though "triple sulphas" and gantrasin are popular also. Sulphadiazine has proved to be a practical agent in clearing meningococcus carriers in closed populations such as barracks and institutions (20).

From his experience with the Research Foundation of Children's Hospital D.C., Ross (75) has found sulphadiazine alone to give excellent results in most cases of meningococcus infection. When response to sulphadiazine is poor he recommends supplementing it with penicillin, or changing to the broad spectrum antibiotics (chloramphenicol and the tetracyclines).

"Resistance" of meningococci to sulphonamides and antibiotics is of two kinds which are quite distinct from each other. One is an innate variation in susceptibility which the organisms naturally have before there has been any contact with drugs or antibiotics, and the other is an acquired "resistance" which follows exposure to the agents in question. A discussion of innate resistance of meningococci may well begin by referring to the comments made by Davis and McDermott (23) that "susceptibility and resistance are quantitative rather than qualitative attributes" and that each agent can inhibit practically all kinds of bacteria if used in high enough concentration, though such inhibition may not involve the usual action of the drug and may have no chemotherapeutic value. Thus it is the degree of resistance with which this discussion is now concerned.

During 1953–54 studies of the response of meningococci to a number of agents were made concurrently by the Children's Hospital in Washington, D. C., the Boston City Hospital, and the National Institutes of Health in Bethesda, Maryland. Many of the same strains were included in all three studies, and similar results have been obtained using widely different techniques.

Table II presents a summary of preliminary tests of relative susceptibility to six antibiotics and two sulphonamides, done with newly isolated strains from sporadic cases of meningococcus meningitis at the Children's Hospital. Although the paper disk technique employed in these tests does not have the

TABLE II

SUSCEPTIBILITY OF NEWLY ISOLATED MENINGOCOCCI TO THERAPEUTIC AGENTS AS DETERMINED BY THE PAPER DISK TECHNIQUE AT THE CHILDREN'S HOSPITAL, WASHINGTON, D. C. *

Agent	Number tests	Number V.S.	Number M.S.	Number S.S.	Number R.
Penicillin	33	25	1	4	3
Aureomycin	33	33			
Chloramphenicol	33	33			
Terramycin	33	33			
Streptomycin	30	5	15	7	3
Bacitracin	33	5	7	13	8
Sulphadiazine	28	21	0	2	5
Sulphamerazine	28	20	1	2	5

V.S.—very sensitive; M.S.—moderately sensitive; S.S.—slightly sensitive; R.—resistant.
** Unpublished data obtained from Drs. E. Clarence Rice and Grace H. Guin; tests performed by Mrs. Anne Stearns.*

quantitative accuracy of some other more elaborate methods it does indicate certain degrees of response. All of 33 strains were "very sensitive" (V.S.) to aureomycin, chloramphenicol, and terramycin; 25 were "very sensitive" to penicillin. They showed less sensitivity to streptomycin, and least to bacitracin; with these last two agents there was considerable difference in response among individual strains.

These same meningococcus strains were included among others in the study made in Boston by Love and Finland (48) with 11 antibiotics and sulphadiazine, using a solid agar medium culture technique. They found that, weight for weight of the antibiotics, penicillin was the most active agent, and polymyxin and bacitracin the least. For each of the broad spectrum antibiotics, there was a relatively uniform susceptibility of all strains, the minimum inhibiting concentrations falling into a narrow range.

The story runs differently in respect to the sulphonamides. In 1940 Branham (9) reported variations in experimental infections in mice. Mice were injected intraperitoneally with a standardized dose of meningococci of maximum virulence suspended in mucin. The sulphonamides were then fed intragastrically two hours later. In these experiments an eightfold variation was found among the strains used. The difference was not correlated with any serological type. These earlier sulphonamides have been supplanted by newer and more effective forms. During the more recent observations similar results have been obtained. Table II shows that Children's Hospital found about one-fourth of their strains to show some resistance to sulphadiazine and sulphamerizine. Love and Finland (48), using sodium sulphadiazine,

TABLE III

VARIATION IN RESPONSE TO SULPHADIAZINE FOUND IN 23 NEWLY ISOLATED MENINGOCOCCUS STRAINS *

Total number strains tested	Minimum mgm. sulphadiazine giving 50% protection in mice against 100,000 LD/50						
	.005	.01	.015	.02	.025	.04	.08
23	7	7	1	2	2	2	2

* *Unpublished data from the laboratory of Dr. S. E. Branham (NIH).*

found a wide variation in susceptibility of their meningococci, a range in minimum inhibiting concentrations of 0.4 to 400 μgm. per ml. Results obtained in Branham's laboratory at the National Institutes of Health are shown in Table III. In this study she employed the same mouse protection technique described in 1940 (9)*. A 16-fold range in dosage was found. These meningococci were all from sporadic cases and were about evenly divided serologically between Groups B and C.

Results obtained with the individual strains that were included in all these studies were interestingly comparable in view of the differences in technique employed. The widest differences in resistance were found by the Boston group. This innate resistance of occasional strains to sulphadiazine can be met by supplementary therapy, if tests are made early enough to reveal it, and such difference in susceptibility by no means detracts from its usefulness as a drug of choice in meningococcus infections.

During the epidemic years of 1941–1943, Phair and Schoenbach (67) studied the response of 430 strains of meningococcus to sulphadiazine, incorporating the drug in starch casein hydrolyzate medium, both as broth and as agar. Of these 98% were inhibited by a concentration of 0.5 mgm. per 100 ml.; only nine strains were resistant.

Love and Finland have suggested that the discrepancy between their results and the earlier findings of Phair and Schoenbach might be due to the appearance of strains of increased resistance in the interval between these two studies. Such an acquired trait is indeed a possibility. However, Branham's earlier 1940 report on work done in 1938 and 1939 showed definite differences in resistance before sulphonamides were in general use.

It seems to the writer that these differences in results may be logically explained on an epidemiological basis. The study made by Phair and Schoenbach was made during a widespread epidemic and 87% of the meningococcus cultures included were of serological Group A; this group was responsible for the epidemic and the cultures were probably much alike, possibly even

* *Pure line mice, 16–20 gm. in weight, standardized mucin suspensions, and five hour cultures of the meningococci of "maximum virulence" were used. "Maximum virulence" means that 2–10 meningococci in 1 ml. of standard mucin suspension would kill a mouse weighing 18–20 gm. in 48 hr. The sulphadiazine was suspended in acacia and administered intragastrically by means of a small catheter. The drug was given two hours after the culture suspension.*

coming originally from the same strain. Hence they responded alike to sulphadiazine. The studies of Branham and of Love and Finland were made during non-epidemic times and the meningococci examined were strains from isolated sporadic cases with no relation to each other, hence with a more individual response to sulphonamides. The writer does not intend to imply that innate resistance to sulphonamides is related to serological group. All of the studies quoted state that no differences in resistance could be correlated with specific groups. The 98% of susceptible meningococcus cultures described by Phair and Schoenbach were not necessarily susceptible because most were of Group A, but because during an epidemic the organisms tend to be alike, often showing an absorption pattern that defines a type within a Group (7). Conversely the resistant strains found by Branham and by Love and Finland were not resistant because most of them were of Groups B and C (only three of A in each study) but because they were sporadic strains, each with its own sensitivity.

Acquired resistance to therapeutic agents has been reported *in vivo* and *in vitro* for a number of microorganisms. Various theories for its development have been suggested, probably the simplest being that the individual cells of any given culture vary in resistance just as they vary in other properties. The most sensitive cells are readily killed allowing the resistant ones to survive a sublethal dose. By such a selective process, all cells in a bacterial population may become more and more resistant. Actually "selection" is a more accurate term than "resistance" to apply to this phenomenon. Very little special study has been made of acquired resistance to sulphonamides by the meningococcus, but with the gonococcus it is well known to occur (85). Miller and Bohnhoff (56) raised the resistance to penicillin of a strain of meningococcus 170-fold during 61 passages in mice treated with subcurative doses of this drug. Development of resistance to penicillin is gradual and "stepwise." They found this resistance to diminish at approximately the same rate at which it was acquired (53). Aureomycin, chloramphenicol, and doubtless other broad spectrum antibiotics, follow the same stepwise pattern as penicillin in developing resistance.

Quite different from any of these agents is streptomycin. Miller and Bohnhoff (52) found that meningococci and gonococci could acquire resistance to this agent with such amazing rapidity as to suggest mutation, and could grow, after two or three transfers, on media containing 50,000 μgm. per ml. Not only was this marked resistance quickly acquired by the meningococcus, but mutants appeared which were actually dependent upon the presence of streptomycin for growth (54). This same phenomenon has been found to occur with streptomycin in several other different bacterial groups. This has been demonstrated with meningococci *in vitro* and *in vivo*, (54, 55, 57, 58). In view of the number of therapeutic agents available for the treatment of meningococcus meningitis it would seem that the use of streptomycin in such infections is contraindicated.

Transduction and Genetic Recombination

As defined by Lederberg (43) genetic recombination includes any process in which genetic factors from two or more parents are combined within one cell. In genetic transduction the hereditary factor may be a very small fragment of a cell, or even of a chromosome. Such a procedure was accomplished as early as 1928 by Griffith (36) who transformed rough (R) avirulent pneumococci of Type II into smooth (S) virulent encapsulated cells of Type I by injecting heat killed virulent S culture of Type I subcutaneously into mice together with an attenuated R strain derived from Type II.

In 1944 Avery and his co-workers (2) showed that the fundamental unit of the active transforming principle was a nucleic acid of the desoxyribose type. They obtained it in a highly purified form and found that a minute amount, under suitable conditions, could induce the transformation of Type II (R) into fully encapsulated Type III (S).

Such transformation has been applied to the meningococcus and to *Haemophilus influenzae* by Alexander and her co-workers. By using desoxyribonucleic acid which they derived from type specific smooth meningococci, Alexander and Redman (1) have been able to induce specific A (Group I) traits in populations of both R and S Group C (II α) cultures; and to induce Group C (II α) specificity in Group C (R, II α) cells. This was accomplished *in vitro* in two ways; (*a*) by exposure of the recipient cells to the desoxyribonucleic acid during their period of growth and (*b*) by exposure of susceptible cells to desoxyribonucleic acid for 15 min., with subsequent destruction of this agent by the addition of desoxyribonuclease. This definite change in Group was demonstrated by capsule swelling and by precipitation of the soluble substance with specific antisera.

This induced change of serological Group is clearly an example of transduction which is actually a form of genetic recombination when this latter term is used in its broad sense.

In the case of the meningococcus the genetic fragment to be transferred is the desoxyribonucleic acid extracted from the parent cell. In order that the R meningococcus cells be able to receive and utilize this genetic factor they must still have within, as pointed out by Griffith 28 years ago, the specific structure which enables the cell to form the capsular substance responsible for the change. Hence all strains are probably not capable of transduction. Alexander estimated that about 1 in every 10,000 cells in the strains of meningococci which she studied were susceptible.

Other methods of inducing genetic recombination have been employed with other groups of bacteria. Some involve conjugation of whole cells with subsequent separation; as i.e. the experiments of Lederberg and Tatum (45, 46, 83) with *E. coli*. In several other organisms bacteriophage has served as the genetic factor (33, 37, 44, 65, 86). Neither of these two methods of inducing genetic recombination has been reported with meningococci to the writer's knowledge.

Genetic transduction in meningococci has much significance for the taxonomist, but has its greatest importance in epidemiology. The possibilities conjured up in this field by an imaginative mind are breathtaking. The role of this phenomenon in the sudden appearence of virulent strains in sharp epidemics has given food for speculation since first discussed in the report of Griffith.

Knowledge of the meningococcus has come on a long journey since the organism was first isolated by Weichselbaum in 1887. It has not travelled steadily and slowly like the tortoise, but has gone in brilliant leaps and spurts, with loitering sometimes along the way, rather more like the hare. And there is much more to be learned about it.

Probably the most urgent meningococcus problem at the present is a clinical one; that of saving the patient exhibiting the Waterhouse–Friderichsen syndrome. In spite of the helpfulness of cortical extracts in preventing adrenal damage, many patients still die too quickly for any kind of treatment to be given, even though the responsible organisms may be found later to be highly sensitive to all of the therapeutic agents in common use. There was a time when all such patients died, so that recovery of a few today is a hopeful sign.

Perhaps the best hope for the future progress of knowledge of the meningococcus lies in a more conscious effect to correlate that which is known, and not to allow a lag of interest just because no epidemic is at hand.

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THE STAPHYLOCOCCUS: SEVEN DECADES OF RESEARCH (1885–1955)[1]

By C. E. Dolman

Abstract

The development of researches into *Staphylococcus pyogenes* is viewed in historical perspective at decade intervals over the period 1885–1955. Beginning when Lister's antiseptic spray ritual was about to be abandoned, the sequence of discoveries and attitudes is traced full cycle to the impending disenchantment with bacteriostatic agents. The early recognition of staphylococcal toxins was submerged by Metchnikoff's theories on phagocytosis, and Wright's on opsonins. The Bundaberg disaster drew attention again to the exotoxins; but almost simultaneously, Fleming's earlier work on the damaging effects of chemical antiseptics upon leucocytes culminated fortuitously in his discovery of penicillin. Before the antigenic properties of staphylococcus toxins could be exploited in a thorough clinical trial of toxoid and antitoxin, the sulphonamide and antibiotic era was upon us. Indiscriminate use of these agents inevitably provoked resistance to them by this versatile and ubiquitous pathogen, and led to the present mounting alarm at the intractability and prevalence of staphylococcal infections.

Among the more chastening chapters in the annals of microbiological research is the story of our apparently dismal failure to control the depredations of the staphylococcus. Repeatedly, fresh light has been shed upon the habits and habitats of these minute clustered spherules, and once-outmoded hypotheses about their metabolic mechanisms have been refurbished. Yet three-quarters of a century after Koch first noted their presence in pus, the staphylococci (like Francis Thompson's angels) "keep their ancient places", no less ubiquitous but still elusive, and (like Lucifer at least) shockingly endowed with apparently new, malign propensities.

The agents responsible for much more sinister threats to human health, e.g. smallpox, yellow fever, diphtheria, malaria, plague, typhus and typhoid fevers, and syphilis, have been largely brought under control and shorn of their former terrors, through such means as immunization of the prospective host, the destruction of vectors, the selective sterilization of transmitting media by chemical or physical means, and antibiotic therapy for already infected patients. But these measures, when variously applied to the control of staphylococcal infections, have been found wanting. Indeed, in many countries nowadays, hospitalization seems to entail a special risk of complications from such long-recognized conditions as puerperal mastitis, pemphigus neonatorum, and postoperative wound infections—according to the type of patient. Moreover, some hitherto unfamiliar clinical entities, such as staphylococcal pneumonia and enterocolitis, appear to be emerging, with derivations often as baffling as their outcome is devastating. A situation

[1]*Manuscript received October 24, 1955.*
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so challenging seems to invite a brief review of main landmarks in the panorama of staphylococcus researches. This will take the form of a brief conspectus at decade intervals over the last 70 years.

1885: Listerian Antisepsis

By 1885, Lister's prolonged researches into the causes and prevention of wound sepsis had won him the acclaim of Europe. Twenty years earlier, Semmelweis had died in a mental hospital, brokenhearted at the scorn heaped upon his doctrines about puerperal sepsis; and Lister, having been introduced to the work and writings of Pasteur, began to develop the theory that air-borne microbes, which the latter had shown were responsible for fermentation and decomposition of beer and wine, might also cause suppuration and putrefaction of wounds.

Lister's accomplishments stand out against a background of hopes first uplifted by the introduction of anaesthetics, then shattered by the ghastly mortality from sepsis in the lengthier and graver surgical undertakings which chloroform and ether made possible. Indeed, to quote Allbutt, "Suppuration, phagedaena, and septic poisonings of the system carried away even the most promising patients and followed even trifling operations. Often, too, these diseases rose to the height of epidemic pestilences, so that patients, however extreme their need, dreaded the name of hospital, and the most skilful surgeons distrusted their own craft. New hospitals or new wards were built, yet after a very short time the new became as pestiferous as the old; and even scrupulous care in ventilation and housemaids' cleanliness failed to prevent the devastation. Surgery had enlarged its freedom, but only to find the weight of its own responsibilities more than it could bear" (1). Or, as von Volkmann wrote, when a surgeon closed a wound, he was "like a husbandman, who having sown his field, waits with resignation for what the harvest may bring, and reaps it, fully conscious of his own impotence against the elemental powers, which may pour down on him rain, hurricane and hailstorm" (28).

Lister was always striving to improve his antiseptic and aseptic techniques, but would not abandon a device until convinced it should be supplanted. Then he was ready to admit his error. For example, at the time of the 1880 International Medical Congress, Lister's confidence was already waning in the dilute carbolic acid spray under which he and his disciples customarily operated; and his address to the gathering was mainly devoted to experimental observations on the healing of wounds, the antimicrobial properties of healthy animal tissues, and the protective role of blood clots peopled with migratory corpuscles. Yet in 1885 Lister was still using the spray, although its efficacy was widely challenged, and he had conceded that "the unconscious caretaker" might be wasted upon a relatively innocuous atmospheric flora. He finally gave up the spray in 1887, and told the 1890 International Medical Congress, "I feel ashamed that I should ever have recommended it . . ." (28).

Meanwhile, others had been investigating the sources of sepsis along different fronts. Ogston (46) had demonstrated in 1882 that "acute and

pyaemic abscesses always contain micrococci", consisting either of "chains interwoven with each other" or of groups "like the roe of a fish", and soon afterwards had named the latter *Staphylococcus*. In 1884, Rosenbach (48), isolated *Staphylococcus* in pure culture, and showed that it was commonly involved in wound suppuration, and was the causal agent of osteomyelitis. The year before, Metchnikoff had published his first report on phagocytosis (40). Thus, by 1885, the provocators and the functions of pus were under study, and the chief defenders of the animal body against an important invader had been revealed.

1895: Leucocytes and Leucocidin

In 1895, the civilized world mourned the death of Louis Pasteur, and his great eulogist, Sir Joseph Lister, became President of the Royal Society. By the shores of Southampton Water, Almroth Wright, Professor of Pathology at the Army Medical School, set about discovering a means of protecting the British Army from typhoid fever by immunization. His future disciple and successor, Alexander Fleming, then a lad of 14, left the seclusion of an Ayrshire village, and made his way to London. Meanwhile, Metchnikoff's researches on phagocytosis had evolved into a theory that inflammation was a biological defence mechanism (39). Any agent harmful to the polynuclear leucocytes would thus be inimical to the host. Van de Velde (52) had recently reported the presence of such an agent, which he termed *leucocidin*, in culture filtrates of *Staphylococcus pyogenes*, and in the exudate provoked in rabbits by intrapleural injection of live staphylococci. This substance caused marked degenerative changes in rabbit leucocytes *in vitro*, and when injected into rabbits, provoked the development of circulating antileucocidin (10).

1905: Antitoxin and Opsonin

By 1905, many of the toxic properties of staphylococcal filtrates had been explored, mostly in German laboratories. Von Lingelsheim (34) had reviewed the aetiology and therapy of staphylococcal infections, and reported the necrotizing and lethal effects of what he first called the *toxin* of staphylococci. Kraus (33) had observed the haemolytic action of certain staphylococcal cultures, and the varied susceptibilities thereto of different species of animal erythrocytes. Neisser (41) had noted the presence of antistaphylolysin in the blood of sick and healthy persons; while he and Wechsberg (42) had described the production of haemolysin and leucocidin, the assay of anti-haemolysin and antileucocidin in human or animal sera, and the pathological changes induced in the kidneys of experimental animals injected with staphylotoxin. The foundations of our present knowledge of staphylococcus toxins and antitoxins were in fact laid half a century ago.

The failure to apply such important findings was partly due to the powerful diversionary influence now emanating from Wright's laboratory at St. Mary's Hospital, London. Wright was so obsessed with "the bankruptcy of medicine" that in 1905 he appealed to the public in a newspaper article entitled "The

World's Greatest Problem", which stressed the need for more and better medical research (8). The pundits of Harley Street were particularly unenthusiastic about his culminating sentence: "That scientific knowledge which alone can avail in the conflict with disease is—practically all of it—still to seek". Nobody could deny that throughout the 40 working years left to him after penning these words, Wright sought manfully to make medicine solvent. In his view, the physician of the future would be an immunizator, aiming to call up the latent forces of the organism, especially through inoculations of heat-killed vaccines. With Douglas (57), he had revealed an important connection between humoral and cellular immunity mechanisms. The brilliant vigor of Wright's efforts to reinforce this link between his opsonins and Metchnikoff's phagocytes blinded him and many of his contemporaries to technical and dialectic fallacies. Yet one of his last papers contained a remarkable recantation of some of the presuppositions on which the rationale of opsonic index determinations was based (55).

Certain strains of *Staphylococcus* proved, perhaps unfortunately, very suitable for these opsonic experiments. Their phagocytosis was so undeniably evident *in vitro* that any observer could be led to dismiss as irrelevant such toxic metabolites as e.g. the leucocidin, whose deleterious effects upon phagocytosis in staphylococcal infections might far outweigh any opsonic benefits accruing from vaccine therapy.

1915: War Wound Sepsis

In the foreground now is the carnage of World War I. By 1915, Wright and a team of devoted associates from the Inoculation Department at St. Mary's, including Fleming, Leonard Colebrook, and John Freeman, were already immured in problems of wound sepsis. Gas gangrene and tetanus took a fearful toll, and all varieties of pyogenic microbes wrought havoc, so that the Surgeon General was moved to declare, "We have, in this war, gone straight back to all the septic infections of the Middle Ages". But on the whole, staphylococci were found too much everywhere to be reckoned a major menace. Wright launched a campaign to deter surgeons from stuffing wounds with chemicals, which not only destroyed leucocytes, but hindered their access to the microbes. "Leucocytes are the best antiseptic" was his dictum, and wound treatment must provide optimal conditions for their functioning (8). Wright's wartime experiences only confirmed his views on the paramount importance of the healthy leucocyte in the bodily defence against infection, whether by Welch's bacillus or by staphylococci. He never altered this belief.

In 1914, little noticed and long overlooked, two papers had appeared, which illustrated the versatility of *Staphylococcus*. Nicolle and Césari (43), reported from the Pasteur Institute that staphylococcal strains isolated from infected farm animals closely resembled cultures from human lesions, even in respect of the necrotizing and lethal properties for rabbits and guinea pigs of their

filtrates. On the other side of the world, Barber (3) attributed sporadic attacks of gastroenteritis affecting visitors to a farm in the Philippines to the presence of staphylococci in the milk of one particular cow.

1925: Denunciation of Intravenous Antiseptics

During the war and postwar years, the disappointing failure of old and new antiseptics to cleanse deep wounds, and the contrastingly "laudable" properties of fresh pus, were analyzed and accounted for in many ingenious ways, by Wright and Fleming. The latter adapted Wright's slide cell device (56) to the testing of chemical antiseptics in the presence of infected blood, and showed that all such agents then available destroyed the phagocytic power of the leucocytes more readily than they killed the microbes present in the mixture (25). In these slide cell experiments, Fleming customarily used a strain of *Staphylococcus* as test organism. The principles thus demonstrated helped to discountenance the prevailing tendency to administer antiseptics intravenously in desperate cases of staphylococcaemia. But, on the whole, progress seems to have been halting or marking time during this period. The march of events in the following decade more than made up for this.

1935: Bundaberg and Penicillin

The death knell of antiseptic therapy appeared to be tolling in 1925; but soon, trumpets were sounding from several directions. By an irony of circumstance, Fleming's tentative clarion call in 1929 was to carry the farthest and echo most. Persons fortunate enough to have been working with Fleming at the time of penicillin's discovery, or shortly after, will recollect the almost childlike eagerness with which he displayed the famous mold-contaminated plate to "The Old Man" (Sir Almroth Wright), to colleagues, and to students. He hoped for great things of this inhibitory factor, particularly in the treatment of boils and carbuncles—for was it not a *Staphylococcus* culture that showed lysis near the fungal growth?—and filtrates were applied topically in a few cases. Despite ribald and derisive comments from The Old Man and others, administration by mouth was also considered. One recalls, for example, helping to make a batch of "mould juice" (as it was irreverently termed in Fleming's laboratory 25 years ago) for administration to an elderly arthritic, whose affliction was held due to enterococci. The patient's bravery in swallowing three pints of the beverage was not rewarded by beneficial results. The erratic potencies and lability of these early crude preparations led to the setting aside of penicillin as a therapeutic agent. Besides, alternative possibilities for dealing with staphylococcal infections were discernible on the horizon.

In 1928, at Bundaberg, Queensland, 12 children had died within 15 to 34 hours of receiving an injection of diphtheria toxin–antitoxin mixture to which no preservative had been added. Among those appointed to investigate the disaster was Burnet, a young bacteriologist from Melbourne, who concluded

that the mixture had become contaminated with a toxin-producing *Staphylococcus* (31). In 1930, soon after the appearance of the first of several papers by Burnet on the production and properties of this formidable exotoxin (4), Professor Fleming suggested to the present writer that this agent, properly detoxified, "might be useful in the boil clinic" at St. Mary's Hospital. In 1931, emigration to Canada interrupted the enquiry thus instigated; but enough had been done to justify continuing *Staphylococcus* researches in the Connaught Laboratories at Toronto.

By 1935, many reports on staphylococcus toxin, toxoid, and antitoxin had issued from laboratories in Australia, Canada, and England. These established the conditions for *in vitro* production of highly potent toxin (5, 12); for the formol detoxification of the product (6, 19); for titrating the combining power of the toxoid (19); and for assaying the antitoxin in terms of an International Standard Unit (29). Arrhenius' comment of many years before, that "staphylolysin behaves in a very peculiar manner", was soon amply confirmed; and the haemolytic effects produced by staphylococcal cultures on blood agar plates, or by their filtrates on erythrocyte suspensions, were recognized as due to multiple factors, even yet not all elucidated (13). In particular, the so-called β-toxin was differentiated from the necrotizing and acutely lethal α-toxin (27). In yet another direction, Jordan, Dack, and others at Chicago confirmed and extended Barber's observations of nearly 20 years before by showing that filtrates from appropriate strains of *Staphylococcus* could provoke symptoms of acute gastroenteritis within a few hours of their ingestion by human volunteers (9, 30). In Toronto, this food poisoning factor, or enterotoxin, was shown to be distinct from the α-toxin (16).

Clinicians were not kept waiting for all the unfolding laboratory complexities to be resolved before having opportunities to try out the toxoid and antitoxin in their patients. By 1935, toxoid had given encouraging results in localized staphylococcal infections (14, 17); while antitoxin had apparently aided recovery in severe and even desperate cases of acute and generalized infection, treated in Toronto and Montreal—the latter group under the supervision of Professor E. G. D. Murray (15).

Numerous difficulties were encountered in the manufacture and administration of these products; in the unravelling of the immunological principles involved; and in the selection of patients, the provision of controls, and the assessment of results. Some physicians could not understand how patients suffering from intractable furunculosis often displayed little or no increase in circulating staphylococcus antitoxin, whereas a few small subcutaneous injections of toxoid usually raised the antitoxin titer and brought an end to the boils. Many surgeons expected small dosages of antitoxic serum to be effective in patients riddled with abscesses, in sites perhaps as inaccessible to the circulating antitoxin as to their pus-letting scalpel; or hoped that large amounts of antitoxin might still avail in patients whose vital centers and viscera were irreparably damaged already by toxins. But in the midst of efforts to clarify the resulting confusion, suddenly there

seemed no purpose in trying. For Dogmak's discovery of the chemotherapeutic properties of the dye Prontonsil in 1935, and the quick identification of the active portion of its molecule, ushered in the sulphonamide era.

1945: Apotheosis of Bacteriostatic Agents

Admittedly, the bacteriostatic agents have proved in many respects a great boon to medicine; but when used irresponsibly or unscientifically—on the principle that pills are much easier to take and administer than antigens and antitoxins—they may do more harm than the intravenous germicides of earlier decades. Excessive amounts can be as toxic for leucocytes, kidney, and liver as any of the older antiseptics; while in smaller, sustained dosages, they may interfere with the host's immunity mechanisms, and induce heritable drug resistance in the invading microbes. Nor can these drugs affect toxin already produced, or still to be produced, by microorganisms surviving in the tissues. Although these handicaps of the new chemotherapy were predictable or soon observable, they are not easily remediable. Just as the time seemed ripe for thoroughly testing the theoretical advantages of combining bacteriostasis and antitoxic immunity, World War II broke out.

This time, the desperate cry for a remedy for the wound infection problems of earlier wars brought forth a wonderful response. Soon after Florey and Chain had shown at Oxford in 1940 how penicillin could be concentrated, the manufacture of this antibiotic had top priorities; and by 1945, the golden droplets of "mould juice" gleaned so uncertainly from the gray-green fungus in small flasks at Paddington 15 years before were being harvested from giant vats in North America at the rate of thousands of gallons weekly. The activity of the product was still assayed against a susceptible strain of *Staphylococcus.*

Waksman's discovery of streptomycin in 1943 began to raise expectations in some quarters that if enough trained technicians diligently searched the soil, other antibiotics might soon emerge to master all bacterial pathogens insusceptible to penicillin and streptomycin. There were many who even contended that bacteriology had seen its heyday, and that enterprising microbiologists should turn to the viruses. In such a climate one could still prophesy; but to stress the role of toxins in staphylococcal infections was like crying in the wilderness.

On the other hand, staphylococcus food poisoning was obviously not amenable to antibiotics, and by now had become recognized in North America and (more tardily) in Great Britain as a very common entity. The wartime prevalence of food-borne outbreaks of acute gastroenteritis afforded an incentive for further exploration of this problem. From the outset, a major difficulty in working with staphylococcal toxins has been to sort out the respective properties of the various haemolysins, and especially to differentiate between their effects and those of the enterotoxin on experimental animals. Now, about 10 years after the enterotoxin had been distinguished from the α-toxin by ingestion experiments on human volunteers, its separate identity

from the β-toxin was likewise demonstrated (18). As this method of testing is too inconvenient and hazardous to be warranted except to settle crucial questions, the kitten test for enterotoxin (21) was also re-examined. The need to avoid false positive reactions due to β-toxin was stressed, and minor modifications in the test were suggested. In the nearly 20 years since the feline reaction was first described, various alternatives have been proposed – involving monkeys, piglets, chicks, baby mice, decapitated frogs, and isolated portions of rabbit intestine—but all proved disappointing. Some imperfections of specificity and sensitivity probably remain inherent in the kitten or cat test for enterotoxin; but recently it has been favorably reappraised by Matheson and Thatcher (38).

The complexity of *Staphylococcus* was again exemplified in 1938 by the identification of a distinct γ-haemolysin in filtrates of certain strains (51). In the next decade, this analysis was to be carried further, and the existence of δ and ε-lysins postulated (53, 36, 23). The apparent nephrotoxic properties of the β-toxin (20), the dermonecrotic action of the δ-toxin (36), and the antigenicity of the specific coagulase (47), were to re-emphasize to manufacturers of staphylococcal antigens the importance of selecting polyvalent strains.

Finally, at the close of the decade, Wilson and Atkinson (54) developed a method of typing staphylococci by means of bacteriophage, first described in 1942 by Fisk (24), which was soon to prove most useful epidemiologically.

1955: Disenchantment and Dismay

This last decade began full of confidence that *Staphylococcus* as a menace to man would soon be laid low. The delusion has been rudely dispelled. Today, staphylococcal infections appear more prevalent, more virulent, and more unavoidable, than they were a quarter-century ago, in the prepenicillin area. Moreover, these infections seem to have assumed the status of an occupational or environmental disease in places where one might least expect to find them, namely hospitals. The final touch of tragic irony in the dénouement is that whereas penicillin–resistant strains of *Staphylococcus* were seldom encountered when supplies of this antibiotic first became unrestricted soon after the war, the majority of hospital strains are now resistant to it.

The current incidence and types of staphylococcal infections inside and outside hospitals are difficult to assess, if only because hospital administrators, and the medical and surgical staffs, display attitudes ranging from that of the alarmist who regards a minute pustule on a baby's bottom as significant, to that of the escapist who avoids admitting the existence of any situation serious enough to involve his institution in possible discredit or a damage suit. To attempt statistically valid comparisons between the situation now and that prevailing a generation or even a decade ago would be manifestly futile. The incidence of staphylococcal infection, calculated as a percentage of total admissions to hospital, or of total surgical operations, may have increased only in relation to all other types of infection. Nevertheless, we must acknowledge the widespread impression of a recent change for the worse

in hospital experience with staphylococcal infections, which is only partly reflected in the medical literature. Troublesome though seldom fatal outbreaks among infants and nursing mothers have brought closure of maternity wards; while anyone professionally connected with a large hospital can recount tragic instances of fulminating infection. For example, a patient hospitalized for pneumococcal pneumonia, and recovering therefrom, died a few days later of staphylococcal pneumonia. A simple appendectomy in a healthy young man (a physician) resulted in septic thrombosis, osteomyelitis of vertebrae, extradural abscess, and paraplegia; while paronychia in a healthy young woman (also a physician) ended within a week in fatal staphylococcal meningitis. These things should not be; yet such examples, culled from local sources, could be matched and multiplied elsewhere in North America, and in the British Isles, Scandinavia, or Australasia. The situation is superficially reminiscent of that prevailing almost a century ago, when Florence Nightingale commented: "It may seem a strange principle to enunciate as the very first requirement in a Hospital that it should do the sick no harm" (44).

These final paragraphs may fittingly consider how it has come about that the latest decade of *Staphylococcus* researches, which dawned so promisingly, should be ending in an atmosphere of somber bafflement. The problem basically involves host–parasite relationships, and can therefore be dealt with from the standpoints of the parasite, of the host, and of the modes of transmission. Considering first the parasite, few would dispute that the indiscriminate use of penicillin during the first years after it became generally available was largely responsible for the growing preponderance of resistant strains. Exposure of so ubiquitous and versatile a parasite to such an inhibitory agent—by every route from inunction, irrigation, and inhalation to injection and ingestion—inevitably resulted in a process of natural selection favoring strains which can produce penicillinase. Alternatively or additionally, exposure of staphylococci to penicillin (or other antibiotics) may induce new, resistant mutants to develop. These processes presumably account for the penicillin-resistant staphylococcal flora developing in war wounds after prolonged penicillin treatment, noted already in 1946 by North, Christie, and Rank (45); and for the observations of Rountree and Thomson only three years later (49), that the nasal carrier rate of penicillin–resistant strains of *Staphylococcus* among 200 hospital staff members was 32%, in contrast to 4% in a similar group of blood donors.

Although there is no laboratory evidence that antibiotic-resistance in staphylococci is yet associated with new or exalted toxigenic capacities, this may be due merely to the inadequacy of present methods for detecting all the complex properties involved. Another worrisome possibility is that mutant strains could develop which flourish only in the presence of low concentrations of certain antibiotics.

Closely connected with these contingencies are the alterations in the potential host resulting from use and abuse of antibiotics. The effects of

administering an antibiotic by mouth are by no means confined to inhibition of a susceptible pathogen. Other changes may be provoked in the intestinal flora, so that potential pathogens, normally held in check, can flourish and cause disorder. What holds true for *Candida albicans*, for example, could equally apply to *Staphylococcus*, which is now known (through development of special media) to inhabit some intestines in significant numbers. Such organisms, multiplying unimpeded by antibiotics or by bacterial competitors, might soon elaborate dangerous amounts of the α-, β-, or enterotoxin. Though α- and β-toxins are apparently not absorbed from the normal intestinal tract, they might occasionally traverse an intestinal mucosa denuded of its customary flora. Similar mechanisms could conceivably operate in the upper respiratory tract. The bacteriologist and clinician together should be able to settle eventually whether the acute toxaemias of staphylococcal enterocolitis or bronchopneumonia may thus be explicable. Meanwhile, there is a sound middle course between that of the optimist who believes that every strain of *Staphylococcus* has its Achilles' heel, and must therefore be challenged in succession with every antibiotic weapon available, and that of the fatalist who believes, so to speak, that a patient's number is up if the strain attacking him has his number on it. This course surely includes the intelligent application, with proper laboratory controls, of the principles of both bacteriostasis and antitoxic immunity.

The problems of transmission, i.e. the epidemiology of staphylococcal infections, can only have brief consideration here. The human nose is now accepted as an important reservoir for *Staphylococcus pyogenes*. Attention was first drawn to this point 20 years ago (17), and there are now numerous reports on its significance (35, 26, 11, 50). Again, new light has been thrown on the transfer of staphylococci between the milk of nursing mothers and the alimentary canal of their infants (22); on the rapidity with which the nasopharynx and faeces of infants harbor staphylococci derived from their environment (37); and on the ready conveyance of mastitis to mothers by infants with air-borne staphylococcal infections of the nose and throat (7).

Efforts to cope with the intransigeance and resourcefulness of *Staphylococcus* entail extremely close analyses of hygienic practices in hospitals. The miscellaneous potential sources of infection, e.g. air and dust, bedding and mattresses, baths and laundry baskets, surgeons' gowns and anaesthetic apparatus, must be not only scrutinized bacteriologically, but dealt with appropriately (32, 2). Unfortunately, control problems are much aggravated by the rapid circulation of employees, whose proliferation as a result of shortened working hours and of more specialized medical and nursing care, multiply the chances of contacts between patients and unskilled, partly trained, or careless persons. The situation will eventually yield to a better understanding and a more enlightened imagination on the part of all, including the highest ranks of clinicians, especially when the notion of sheltering under the antibiotic "umbrella" has been discarded as finally as the ritual of Lister's spray.

In the acquisition and dissemination of relevant data, the bacteriologist will doubtless play as full a part in future *Staphylococcus* researches as he has done over the past 70 years. The adversary is formidable, but there is no need for dismay. This review was not intended to point a clear path straight through the woods, but was undertaken in the belief that backward-glancing often clears the eye, adjusts the perspective, and brings encouragement. We have come a long way in what is, after all, only the lifetime of an average man.

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THE RELATIONSHIPS OF THE BACTERIA AND VIRUSES TO OTHER LIVING THINGS[1]

By Robert S. Breed[2]

Abstract

In the forthcoming edition of Bergey's Manual, the classification used has been rearranged on the assumption that the polar-flagellate, photoautotrophic bacteria existent today are the living organisms that are most nearly like primordial types of bacteria. The primordial bacteria—like living things out of which other living things (animal and plant) have developed—must have been preceded by other living things of a size comparable to living viruses. However, unlike filterable viruses, which represent a comparatively recent evolutionary development, primordial living things must have existed on simple, presumably inorganic foods and must have been very different physiologically from viruses. The new classification has been greatly developed by the group of specialists that have helped in the preparation of the new Manual. The (*a*) blue-green algae, (*b*) bacteria, (*c*) rickettsias and viruses are arranged in the improved classification as three classes in Division I, *Protophyta* Sachs of the Plant Kingdom. The number of orders in Class II, *Schizomycetes* has been increased from 5 to 10 by a subdivision of previously recognized orders and by the recognition of a recently proposed new Order X, *Mycoplasmatales* Freundt for the pleuropneumonia-like organisms. The classification of the rickettsias and viruses will be discussed in a separate paper.

The development of the various editions of Bergey's Manual (1, 2, 4) has caused those of us responsible for this work to give much thought to the probable evolutionary development of the simple living things that are included under the general terms bacteria and, more recently, viruses. It is natural to think of these organisms, endowed as they are with a simple structure, as being primordial or primitive in nature. This concept is fundamental in all systems of classification that have been developed for them. Nevertheless it should be kept in mind that the different species or kinds of these simple living things now existent may have undergone much change during the very long period of time in which living things have undergone their evolutionary development. It is difficult to picture the environment under which these undifferentiated, unicellular organisms lived when they first appeared on this earth; but it is certain that this environment was quite different from the environment in which such organisms live today.

One important feature of the present-day environment that would have been lacking in the earliest periods would be association with more highly developed types of living plants and animals with a resultant accumulation of organic materials that must take place as the natural processes of life and death go forward. Saprophytic organisms and, still less, parasitic organisms would not have had conditions favorable for their existence in the earliest periods in which life developed on this planet. This makes it necessary to

[1]*Manuscript received October 17, 1955.*
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assume that the earliest living things must have existed on comparatively simple, largely inorganic food materials. With this thought in mind, some students of the systematic relationships of living things have thought of the chemoautotrophic bacteria that still exist as being more like primordial living things than other types of bacteria that existed before other unicellular plants and animals were developed.

It is true that the chemoautotrophic organisms are able to live on simple inorganic foods that were in all probability available to living things under early conditions in the development of the earth. However, it does not necessarily follow that chemoautotrophic forms are the only ones that could have existed in the beginning. It seems even more reasonable to assume that early living forms developed a pigment similar to chlorophyll that enabled primordial bacteria to utilize the sun's energy in synthesizing organic matter. Such photosynthetic pigments are found in purple and green bacteria. These photoautotrophic forms could have existed on the simple foods available when life began as readily as could chemoautotrophic forms.

In either case, it is necessary to assume that living protoplasm with its complex enzymatic systems was existent before primordial bacteria were able to use inorganic materials as food. In other words, complex proteins were already in existence before either chemoautotrophic or photoautotrophic bacteria of the types now found on the earth could exist.

Even if it is granted that photoautotrophic living things were primordial, it must also be granted that when the existence of such organisms is postulated, we are not starting with the beginning of life itself. So little is known about the possibility of living proteins (protoplasm) developing out of inorganic compounds that speculation regarding this development has brought but very little information that is factual.

In the forthcoming edition of Bergey's Manual, the outline classification has been rearranged on the assumption that the photoautotrophic bacteria existent today presumably are the living organisms that are most nearly like primordial types of bacteria.

In support of this thought it should be kept in mind that the earliest living forms must necessarily have been free-living forms, not saprophytes nor parasites. This being the case, forms such as viruses that are very tiny in size and therefore necessarily of a simple structure ought not to be regarded as primitive just because of a comparatively simple structure. The viruses are adapted to life within living protoplasm and represent an extreme degree of specialization to a parasitic existence. They are known as organisms that invade the living cells of higher plants and animals, including man. The latter are the living things that were latest in development in geological time. Viruses could not have existed before their host plants and animals were developed.

It is not surprising that a great development has taken place in outline classifications since bacteriologists first tried to develop such classifications to express the possible relationships of the organisms with which they have

worked. While O. F. Mueller (24) and C. G. Ehrenberg (13) made simple beginnings along this line, their knowledge of bacteria as they are known today was very limited indeed. Even in 1838 when Ehrenberg published Die Infusionsthierchen als Volkemmene Organismen, microscopes had not yet been developed to a place where even large bacteria could be studied with any satisfaction.

By 1872, Ferdinand Cohn (9), the botanist, began to understand that a great variety of types of bacteria were in existence, and he was able to arrange an outline classification on which later classifications of bacteria have been built. However, his first outline classification of bacteria was scarcely published before he felt that he should have expressed the relationships of the bacteria to the simplest types of algae in a more intimate way. He therefore drew up a second classification in 1875 in which he integrated the known groups of bacteria with known groups of blue-green algae in a class, the *Schizophytae*. This arrangement assumed that the bacteria had a much more intimate relationship to the blue-green algae than the true fungi have to the green, red, and brown algae.

It should be noted that early classifications of bacteria were based primarily upon structural characters, particularly shape of the cells. This was a natural development as morphological characters had been found to be useful in drawing up natural classifications of higher plants and animals. It is also quite natural that workers who drew up these classifications should have regarded the spherical organisms that they found, as being primitive in nature. Little was known at that time of the distribution of bacteria in nature. It was not until later that it came to be realized that the bacteria that are spherical in shape are normally found on the skin or in the skin glands of vertebrates. Few of the cocci exist as free-living forms in water or soil. Likewise, when physiological studies were made, it was found that the cocci require comparatively complex foods for their existence. Few modern classifications retain the arrangement in which cocci are placed first, the arrangement first suggested by Cohn (9).

Others have developed the early classifications* drawn up by Cohn with many individuals contributing to the development of a better and better understanding of the evolutionary development of the bacteria. In the 1890's two groups of individuals undertook the publication of Manuals describing the known species of bacteria. These two groups exercised great influence on the development of systematic bacteriology.

Migula (21, 22) and his students began their work at Karlsruhe, Germany, in the early part of the 1890's, publishing various papers and books, the last of which was his System der Bakterien in 1900 (23). Only one edition was published.

During the same period K. B. Lehmann and R. E. Neumann (20) of Würzburg, Germany, began the publication of their *Bakteriologische Diagnostik*,

**For a more detailed discussion of outline classifications developed by bacteriologists, see Manual, 3rd ed., 1930, 1–23; and Manual, 6th ed., 1948, 5–38.*

the first edition of which appeared in 1896. The publication of the first edition was soon followed by a second and by later editions, the work being seriously interrupted by the first world war after the publication of the fifth edition. Following the war they republished the fifth edition with a supplement, and later carried through a complete revision of this text, which appeared as the seventh edition in 1927. No further editions have been issued.

In the meantime, interest in taxonomic work had crystallized in the newly organized (1899) Society of American Bacteriologists, led at first by F. D. Chester (8), who began his work before 1900. His *Manual of Determinative Bacteriology*, published in 1901, had great influence in guiding the thought of American bacteriologists, but it has never been widely known outside of North America.

As the Society developed, others took an active interest in this work; among them R. E. Buchanan (6), who organized an outline classification of all bacteria as then known. This was published in 1916–18, just as another member of the Society, C.-E. A. Winslow, who had, with his wife, completed a monographic study of the *Coccaceae* (33), urged the Society to organize a Committee to study the development of a better classification for bacteria. The Society of American Bacteriologists' Committee, of which Winslow was made chairman, combined forces with Buchanan and published first a preliminary and then a final report (32) on the classification of bacteria. The report of this Committee was accepted with the thought that further revisions of this outline classification were to be expected as knowledge developed.

Meanwhile in Europe, Orla-Jensen (27) had made notable contributions to knowledge in this field. Still later A. J. Kluyver and C. B. van Niel (18) and others, continued the development of classifications of bacteria, but European workers have been badly handicapped in their work because of the chaotic conditions that have existed during two world wars fought largely in Europe.

Developments in the field of systematic bacteriology before 1920 led to the publication by D. H. Bergey of a manuscript on which he had been working for a long time, his thought being that a new edition of Chester's Manual of Determinative Bacteriology was badly needed, as indeed it was. In order to aid Bergey in securing publication of his manuscript, a Committee was appointed to assist him, by the Society of American Bacteriologists, Dr. F. C. Harrison, Chairman. The first edition of Bergey's Manual (1) appeared in 1923. Successive editions of this Manual were issued in 1925, 1930, and 1936. Before his death in 1937, Bergey requested that an Editorial Board take over future editions of Bergey's Manual.

At the same time, Bergey used the accumulated royalties that had previously been placed in the custody of the Society of American Bacteriologists to organize the so-called Bergey's Manual Trust. The publication of the fifth and sixth editions of Bergey's Manual (2, 4) has been carried out by the Trustees of this Trust, who, by the provisions of the Deed of Trust, must always be men trained as bacteriologists. The Board of Trustees consisted at

first of Dr. D. H. Bergey, Prof. R. S. Breed, and Prof. E. G. D. Murray. Dr. A. Parker Hitchens was elected to this Board after Dr. Bergey's death, when Professor Breed was made Chairman of the Board.

Because of the truly enormous development of our knowledge of bacteria, viruses, and related organisms, the Editorial Board asked students of special groups to assist in the revisions of the groups in which they were interested. Thus more than 40 specialists assisted in the preparation of the fifth edition (2), and more than 60 individuals in the preparation of the sixth edition of the Manual (4). Canadian bacteriologists as well as bacteriologists from the U.S.A. have participated in the Manual work from the beginning. This participation by Canadian workers has increased during the preparation of the manuscript for the seventh edition, as has the participation from other countries. Fourteen countries are represented among the more than 100 specialists that have been working toward the publication of the seventh edition of the Manual.

After the death of Dr. A. Parker Hitchens, Dr. N. R. Smith was appointed to the Board of Trustees and to the Editorial Board of the Manual, and shortly thereafter the Board of Trustees was made a board of five members by the election of Dr. R. E. Buchanan, and Dr. Harold J. Conn to this Board.

In preparing manuscripts for the seventh edition of Bergey's Manual, specialists have found many places where the relationships of described species of bacteria have not been well presented in the literature. Consequently, they have been stimulated to publish many papers reporting their findings. In every case, the individual specialist has been a person who has actively worked with cultures of the organisms that belong to the group for which he has prepared the manuscript. Thus, the development of the new edition of the Manual has stimulated much research in the field of systematic bacteriology that would never have been accomplished under other conditions. It is hoped that in the future the Bergey's Manual Trust can become a center for research in the field of systematic bacteriology and virology. The work thus far accomplished has been carried out largely by volunteer workers. If adequate funds were available for the support of such work, its value could be greatly increased.

Out of studies by specialists of the accumulated knowledge of the systematic relationships of the microorganisms considered in *Bergey's Manual of Determinative Bacteriology*, the three of us chiefly responsible for organizing this work (Prof. R. S. Breed, Prof. E. G. D. Murray, and Dr. N. R. Smith) have developed an outline classification which expresses our ideas of the relationships of the simplest types of living things. These are represented by such common terms as true bacteria, filamentous bacteria, actinomycetes, slime bacteria, spirochetes, rickettsias and related larger viruses, and the filterable viruses. This general classification also expresses our ideas of the relationships of these undifferentiated types of living things to higher plants.

This outline may not express the views of other special students of this subject adequately, as all such outlines represent compromises between differing viewpoints. One such difference of viewpoint that has been discussed among the three of us chiefly responsible for the outline given here has been the question whether a third kingdom to include the *Protophyta*, as defined below, ought not to be recognized in addition to the Plant and Animal Kingdoms. Prof. E. G. D. Murray has been the one in our group who has felt most strongly that the bacteria and related organisms are so different from plants and animals that they should be grouped in a kingdom equal in rank with these kingdoms. It is quite probable that support for this viewpoint would be stronger if early biologists had known how different these important and widely diversified microorganisms are from plants and animals. Even today it must be recognized that our knowledge of the number of kinds of bacteria is growing rapidly as habitats not previously adequately explored are studied. The human body is, as a matter of fact, practically the only habitat that has been comprehensively studied as a source of bacteria. Even in this case it is the bacteria that cause diseases that are best known.

Our knowledge of the still smaller types of parasitic and pathogenic organisms, such as the numerous kinds of organisms found in the *Rickettsiales* and *Virales*, is still more inadequate than our knowledge of the true bacteria. In fact our present-day knowledge of the filterable viruses could perhaps best be compared with Cohn's knowledge of the bacteria when he first drew up a system of classification for bacteria in 1872.

It will be noted that in the outline presented here we have included (*a*) the blue-green algae, (*b*) bacteria and related forms, and (*c*) the rickettsias and viruses in a single division of the plant kingdom, for which the term *Protophyta* has been used. This name was suggested by a botanist, Sachs (30). Recently Sachs' concept of this group has been developed further by a Russian systematist, N. A. Krassilnikov (19), and it will be developed still further in the seventh edition of the Manual.

Of the three names used for the different classes of *Protophyta*, one was suggested by von Nägeli (25) and a second by Cohn (10), and these have been generally used. The development of our knowledge of the rickettsias and viruses is so recent that no truly satisfactory class name has previously been suggested for this entire group. This has made it necessary for Dr. C. B. Philip, who is acting as editor of the section discussed in Supplement I of the sixth edition of the Manual, to suggest a name for this class which is more appropriate for the entire group of organisms included in the orders *Rickettsiales* and *Virales* than any that has previously been suggested. As Dr. Philip has discussed the new developments in the classification of the order *Rickettsiales* in a paper which accompanies this one, the following discussion is limited to an explanation of the reasons for increasing the number of orders recognized in Class II, *Schizomycetes* von Nägeli from 5 to 10.

The organisms placed in Class II, *Schizomycetes* von Nägeli in the sixth edition (4) were arranged in five orders as follows:

Division I. *Schizophyta* Cohn (9) (Fission plants.)
Class I. *Schizophyceae* Cohn (10) (Fission algae. Blue-green algae.)
Class II. *Schizomycetes* von Nägeli (25) (Fission fungi, bacteria.)
Order I. *Eubacteriales* Buchanan (6) (The true bacteria.)
Order II. *Actinomycetales* Buchanan (6) (The mycobacteria, actinomyces, streptomyces and related forms.)
Order III. *Chlamydobacteriales* Buchanan (6) (The alga-like, filamentous bacteria.)
Order IV. *Myxobacteriales* Jahn (17) (The slime bacteria.)
Order V. *Spirochaetales* Buchanan (6) (The spirochetes and related forms.)
Supplement: Groups whose relationships are uncertain.
Group I. Order *Rickettsiales* Buchanan and Buchanan (5) emend. Gieszczykiewicz (15) (Rickettsias and related organisms.)
Group II. Order *Virales* Breed, Murray and Hitchens (3) (Filterable viruses.)
Group III. Family *Borrelomycetaceae* Turner (31) (Pleuropneumonia-like organisms.)

This general outline is like that followed in previous editions of the Manual and is based upon the outline classification developed by Buchanan (6) in 1916–18. It is to be expanded in the seventh edition of the Manual as follows:

Division I. *Protophyta** Sachs (30) emend. Krassilnikov (19) (Primitive plants.)
Class I. *Schizophyceae* Cohn (9) (Blue-green algae.)
Class II. *Schizomycetes* von Nägeli (25) (Bacteria and related forms.)
Order I. *Pseudomonadales* Orla-Jensen (28)
Order II. *Chlamydobacteriales* Buchanan (6)
Order III. New order to include *Hyphomicrobium* and related organisms.
Order IV. *Eubacteriales* Buchanan (6)
Order V. *Actinomycetales* Buchanan (6)
Order VI. *Caryophanales* Peshkoff (29)
Order VII. New order to include *Beggiatoa* and related organisms.
Order VIII. *Myxobacteriales* Jahn (17)
Order IX. *Spirochaetales* Buchanan (6)
Order X *Mycoplasmatales* Freundt (14)
Class III. New class to include rickettsias, viruses and related organisms.
Order I. *Rickettsiales* Buchanan and Buchanan (5) emend. Gieszczykiewicz (15)
Order II. *Virales* Breed, Murray and Hitchens (4)
Division II. *Thallophyta* Endlicher
Division III. *Bryophyta* Haeckel (16)
Division IV. *Pteridophyta* Haeckel (16)
Division V.† *Spermatophyta*

It has been felt desirable to subdivide the Order *Eubacteriales*, as defined in the sixth edition of the Manual, into Order I, *Pseudomonadales*, which includes all of the polar-flagellate types of true bacteria, and Order IV, *Eubacteriales*, which includes the peritrichous types of true bacteria. As in the sixth edition, the photosynthetic purple and green bacteria that are polar-flagellate have been included in the order with the colorless polar-flagellate bacteria. This arrangement emphasizes a concept first introduced into the classification of bacteria by Migula (21). This concept is analogous to the concept on which the orders recognized in *Protozoa* as *Flagellata* and *Infusoria* are based.

**Protophyta was previously used by Endlicher, S. (Genera Plantarum, Vindobonae, 1836, p. 1) in two different senses. 1—For Sectio I, Algae and Lichens of his Regio I Thallophyta. 2—For Cohors II of his Sectio III, Acrobrya to include horsetails, ferns, etc. This use may be disregarded under Article 26 of the International Code of Botanical Nomenclature. This reads: "The rules of priority and typification do not apply to names of taxa above the rank of orders."*

†Goebel, K,, in his edition of Sach's Grundzüge der Systematik und speciellen Pflanzenmorphologie, p. 334, 1882, was apparently the first author to use this name, although he used the incorrect spelling Spermaphyta.

Bacteriologists have recognized differences between polar-flagellate and peritrichous bacteria ever since Migula emphasized them, but there has always been a residual protest against drawing a sharp line between the two groups of bacteria. While there is good reason to draw a sharp line between the ordinary polar-flagellate types of bacteria and the peritrichous types, there are certain groups of bacteria such as legume-nodule bacteria (*Rhizobium*), the violet bacteria (*Chromobacterium*), the agrobacteria (*Agrobacterium*), and certain motile forms placed in the family *Corynebacteriaceae* that have presented forms of peritrichous flagellation that appear to be intermediate between polar and peritrichous flagellation. Some cultures of these organisms are found to show only a single flagellum, while others closely related to these monotrichous species show several flagella peritrichously arranged. On a casual examination these conditions may appear to form a transition between the two types of flagellation. However, this clearly is not the case. This apparently intermediate-type of flagellation seems to be a comparatively recent development in which certain peritrichously flagellated species have undergone a retrogressive specialization. In this the organisms have become primarily dependent on one flagellum as their chief organ of locomotion. They therefore are included in Order IV, *Eubacteriales*, with other peritrichous bacteria. This is here defined to include not only the bacteria that are peritrichously flagellated, but also such non-motile forms as seem by their physiology to be closely related to these peritrichous species.

The placing of non-motile species of bacteria in systems of classification has always caused difficulty. Some students think that lack of motility is a character which should be used as a basis for separation of groups. However, evidence is continually accumulating that shows that separation of larger groups among the bacteria solely on the basis of motility or lack of motility leads to a violent disarrangement of natural groupings. Some non-motile bacteria present fundamental physiologies and other characters that are much like the similar characters of well-known polar-flagellate organisms. Such non-motile species are placed in the classification used here in Order I, *Pseudomonadales*. However, where non-motile species show fundamental physiologies and other characters more like those of peritrichous species, then they have been placed in Order IV, *Eubacteriales*. Organisms living in habitats where they are unable to use organs of locomotion are usually found to be non-motile. This is a natural evolutionary development.

Some bacteria develop into trichomes which may be defined as chains (filaments) of bacteria where the relationship between the cells in the chain have become so intimate that the cells rarely live a separate, independent existence. Sometimes the cells in the chain show a differentiation into holdfast cells and/or reproductive cells distinct from the usual vegetative cells. This differentiation resembles that found among the simpler algae. Because the cells in these trichomes sometimes develop flagella that are placed singly or in a tuft near or at the pole of the cell while others develop cells with peritrichous flagella, it has been felt desirable to recognize two orders among

these trichomatous bacteria: Order II, *Chlamydobacteriales*, for the polar-flagellate types; and Order VI, *Caryophanales*, for the peritrichous and related types.

Little is known about certain species of bacteria which show a budding form of reproduction that is different from the simple cell division (fission) that takes place in the four orders previously discussed. Only a few of these species that reproduce by budding are well known, though some of them occur abundantly in suitable habitats. Because the indications are that many species of these organisms exist in nature, Prof. H. C. Douglas has set these apart in a new order that will be properly described in a later publication. Where flagellation has been observed among these budding forms, it is of the polar type so that Order III has been associated with Order I, *Pseudomonadales* and Order II, *Chlamydobacteriales* in the arrangement of the 10 orders as given above.

Until recently everyone has thought of Order V, *Actinomycetales*, as including species all of which were non-motile. However, Couch (11), in a series of papers, the latest published in 1953, has shown that microorganisms that clearly belong in this order sometimes exist in water rather than in soil or as pathogens affecting animals or plants. These water-inhabiting, saprophytic types of *Actinomycetes* have developed sporangia in which motile or non-motile spores may develop. In a way they are analogous to the so-called water molds. The structure of the vegetative cells and mycelia of these water-inhabiting *Actinomycetes* is clearly like that of the aerobic *Actinomycetes*.

Order VII as recognized in the outline used in the new edition of Bergey's Manual has been newly organized by Dr. R. E. Buchanan to include a group of primarily trichomatous bacteria that are motile but which lack flagella. In spite of this lack they have the power to glide or oscillate as do certain species of blue-green algae. While none of these bacterial types develop photosynthetic pigments, they are frequently and apparently quite properly regarded as colorless, saprophytic forms of blue-green algae. Some oxidize sulphur compounds with the liberation of free sulphur granules that appear in their protoplasm. Some specialists even prefer to transfer this group to the *Schizophyceae* as colorless species of blue-green algae rather than to include them with the bacteria. As bacteriologists have been primarily responsible for developing our knowledge of the species in this order, they are retained here in Class II, *Schizomycetes*. At the proper time and place Dr. Buchanan will publish and describe this order.

Our knowledge of Order VIII, *Myxobacteriales*, the so-called slime bacteria, was first developed by botanists rather than bacteriologists. These organisms occur in leaf mold and on the dung of animals. The cells of these species move with a flexuous motion in a slime which normally grows up into fruiting bodies large enough to be visible to the naked eye.

The organisms placed in Order IX, *Spirochaetales*, have always been set off by themselves though certan species are known that are so much like those

placed in the genus *Spirillum* in Order I, *Pseudomonadales*, that they may be regarded as transitional forms. Sometimes, without sufficient justification, these spirally twisted organisms have been placed among the *Protozoa*.

The 10th order of Class II, *Schizomycetes*, is the newly-organized Order X, *Mycoplasmatales* Freundt. Because a review of the nomenclature of the pleuropneumonia-like organisms (Buchanan, Cowan, and Wikén (7)) has shown that the first generic name applied to these organisms that has a legitimate standing is *Mycoplasma* Nowak (26), this name has been adopted for use in the classification of the pleuropneumonia-like organisms that has been prepared by Freundt (14). This generic name has also been used by Edward (12). While other order names, such as *Borrelomycetales* Turner (31), have been suggested, the generic name *Borrelomyces* Turner, on which this name is founded, has never come into general use, and is in fact an illegitimate homonym of *Mycoplasma* Nowak. Acceptance of the order name *Mycoplasmatales* is in accordance with the principles of the Bacteriological Code of Nomenclature and should tend to stabilize the nomenclature of this group.

In closing this discussion of the revised classification, it should again be emphasized that it has been developed as a result of a study of the ideas which previous workers have expressed in preparing the outlines that they have suggested. Previous workers have laid what appears, with our present knowledge, to be a satisfactory foundation on which to build. The introduction of new ideas has come about largely as a result of continuous study and conference with our colleagues and with the specialists who have contributed and are contributing so much to the knowledge that has accumulated in past years. A generous share of the credit for the things that constitute a real advance in our knowledge should go to these specialists. Where the classification here presented has defects, it is to be hoped that they will be discovered promptly and eliminated.

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"ORDNUNG IN DAS CHAOS" MIGULA[1]

By S. T. Cowan

Abstract

Bacterial classifications based on morphology soon led to chaos, and a semblance of order was restored by using physiological characters. With the accumulation of knowledge chaos is raising its head again. Good classification depends on accurate characterization; standardized methods can help to make descriptions comparable and of more uniform value. Many descriptions need amplification and this could be provided by the expert sub-committees of the International Committee on Bacteriological Nomenclature. Classification must precede the naming of the bacteria, and much remains to be done in the realm of nomenclature. The year 1753 was fixed as the starting date; thus many names without adequate descriptions are in use today. It appears that *Serratia marcescens* was originally applied to a yeast and is thus not available as the name of a bacterium.

Steps that might be taken to reduce nomenclatural chaos include the conservation of certain specific epithets and the selection of type strains; withholding recognition of species for which a type culture is not available; the registration of new names; and publication of newly described species in a limited number of recognized and official journals.

Civilized man, dominated as he is by the Civil Servant, is disposed to collect, keep, and classify, and this is his attitude towards bacteria. How does the bacterium react? Does it behave in an orderly manner and allow itself to be regimented and neatly stowed away, or is it, Irish in temperment and always "agin the government", creating disorder for the poor man who tries to tidy up systematic bacteriology. The word chaos seems particularly applicable to bacterial systematics, but Breed (8) challenges this view and says that we should not accuse Nature of creating chaos. But this is precisely what we are not doing; we describe our own efforts as chaotic, we do not criticize the material we attempt to classify.

The earliest classifications of bacteria were based largely on differences in morphology, but towards the end of the nineteenth century an attempt was made to bring order from chaos ("Ordnung in das Chaos", Migula, (27)) by introducing cultural differences so that only the primary divisions were based on morphology. Each advance in technique gave more power to the elbow of the taxonomists who, to their credit, seldom failed to use the tools provided for them by those whose interests lay in other fields. At first simple cultural characters were used to make the differentiation desired, and most often the distinction was between the pathogenic and the saprophytic; thus Andrewes and Gordon (5) showed a correlation between the pathogenicity of staphylococci and the rate of gelatin liquefaction, but none between pathogenicity and pigmentation. Fermentation reactions, or more correctly the ability to produce acid in media containing certain sugars, were used extensively in early schemes of classification, but with the improvement in serological methods brought about by the absorption technique and qualitative antigenic analysis, schemes based on fermentation fell into disrepute. However, there are fashions in

[1]*Manuscript received October 5, 1955.*
Contribution from the National Collection of Type Cultures, London, United Kingdom.

all things, and in recent years 'sugar' reactions have come into their own and are now being used increasingly in the further subdivision of groups such as *Salmonella* and *Streptococcus* that, a few years ago, were characterized almost exclusively by antigenic pattern. It seems to be inevitable that techniques long since discarded as useless will be rediscovered and used in the latest development of a diagnostic scheme or classification.

At this point a word might be said of diagnostic keys and the part they play in accentuating or reducing the chaos that is bacterial taxonomy. The best known key is to be found in *Bergey's Manual of Determinative Bacteriology* (10), but another developed by Skerman (32) is likely to be more useful to the diagnostician. Both schemes have their drawbacks, and that of the *Manual* has the serious disadvantage that, as the keys to the higher ranks are almost useless, the user must know enough of bacteriology to be able to place his organism in a family, and, in some cases, into a genus. Murray (28) believes that the uselessness of the descriptions of the higher ranks is due to too many units of differing characters being grouped in too few subdivisions. The bacterial dichotomist's pattern is completely upset by gaps in characterization, and his chief bugbear is his inability to supplement the descriptions of species of which a representative has not been maintained in culture. Such 'non-extant' species would be better left out of diagnostic keys, but the bacteriologist seldom has the moral strength to omit them. Skerman's scheme falls down because it attempts to be too comprehensive, and when information is not available, it assumes that a character may be either positive or negative. These keys, based as they are on inadequate data, do not simplify taxonomy, and in some cases can increase confusion by making possible an apparently legitimate diagnosis from an improbable combination of characters.

Mental indigestion follows too plentiful a helping of undigested facts, and too many papers present half-baked results of incomplete experiments; this urge for publication plays its part in adding to confusion. The application of results achieved by newly-developed methods needs caution, and the successful taxonomist may be the one who realizes the possibilities and limitations of a test. Although division based on a single character is undesirable, it may be necessary for want of adequate knowledge; but it is tolerable only as a temporary expedient (8) and the value of the resulting subdivision will be directly related to the acumen of the worker who makes it.

Unnecessary confusion may result from faulty interpretation, or lack of knowledge of earlier work; the validity of *Micrococcus luteus* Cohn and its characterization is a case in point. Cohn's genus *Micrococcus* contained a variety of different organisms, most of them rod-shaped. Argument centers on the ability of *M. luteus* to liquefy gelatin (1, 9, 19) and on the suitability of a proposed type strain, ATCC 398 (9, 16, 19). Breed (9) proposed the strain as type because he believed that it could utilize ammoniacal nitrogen, but that it cannot do (16, 19); Evans and his colleagues (16), ignoring the earlier descriptions and simple nitrogenous requirements, propose recharacterizing the species so that ATCC 398 can be accepted as the type. This seems

to be a good example of making the square peg fit the round hole. Into the associated nomenclatural controversy about *Micrococcus* vs. *Staphylococcus*, another red herring has been introduced, apparently because of unfamiliarity with an out-of-print book. Evans, Bradford, and Niven (16) say "There seems to be a reasonable doubt as to whether the correct specific epithet should be *aureus* or *pyogenes*"; but reference to *Mikro-organismen bei den Wundinfectionskrankheiten des Menchen* shows that Rosenbach proposed the names "Staphylococcus flavus oder aurëus und Staphyl. albus". Describing the sources of the strains, he named them "*Staphylococcus aureus* (mit oder ohne albus)". What Evans and his colleagues overlook is the fact that Rosenbach does not, at least in his book, use *Staphylococcus pyogenes*; consequently that binomial must be attributed to a later author. The only doubt might be whether Rosenbach ever intended to use *Staphylococcus flavus* instead of *Staphylococcus aureus* (14).

The taxonomic abnormalities so far cited are trivial compared with the epileptiform convulsions that periodically afflict the family Enterobacteriaceae. In the good old days (as recent as 1920) the family (then named Bacteriaceae) had only six genera, of which one (*Zopfius*) would now be ejected. In the 1948 edition of Bergey's Manual there were eight genera (*Escherichia*, *Aerobacter*, *Klebsiella*, *Erwinia*, *Serratia*, *Proteus*, *Salmonella*, and *Shigella*), but by 1954 several additional groups were proposed, each approximately equivalent to a genus (*Arizona*, *Bethesda*, *Cloaca*, *Hafnia*, *Providencia*) (24), and the deletion of only one old genus (*Aerobacter*) suggested. Furthermore there were even suggestions (23) that certain *Proteus* species be given generic rank (*Morganella*, *Rettgerella*). Where this process will end is anyone's guess, but what is vitally important is that those most concerned in their identification should aim at a better definition of relations between established genera and species, rather than take the easy way out by creating new ones. That the end of this multiplication of genera is not yet in sight is evident from the absence of a place for motile forms with IMViC reactions $--++$ which fail to qualify for *Klebsiella* and *Cloaca* because they are not capsulated and do not liquefy gelatin.

In the family Enterobacteriaceae, what one worker regards as a species is, to another, a 'group' or genus. Locke in his *Essay concerning human understanding* (25) aptly comments that "*genera* and *species* of things (for those Latin terms signify to me no more than the English word sort) depend on such collections of ideas as men have made, and not on the real nature of things". Bacteriologists, and particularly serologists, need to ponder well on the species concept. "A silent and a striking watch are but one species to those who have but one name for them: but he that has the name *watch* for one, and *clock* for the other, and distinct complex ideas to which those names belong, to *him* they are different species" (25).

The enterobacteriologists seem to be splitters *par excellence*; this is not surprising as they have the tools which enable them to make fine subdivisions. The problem they create is the assignment of rank to the different levels of

their groupings. As epidemiologists and bacteriologists their interests lie in more and more subdivisions, and they are not particularly concerned with the names applied to them. Unfortunately they use names which have at least a superficial resemblance to Linnaean binomials (e.g. *Salmonella typhimurium* and *S. panama*), but in rank these correspond only to serotypes of species in other families (e.g. *Streptococcus pyogenes* types 1 and 2). When strains of *Salmonella* differ only in antigenic structure they are given names that suggest they are separate species; from this it may seem logical that strains differing in biochemical characters should be regarded as being of different genera. This seems to be the only explanation for the wish to create four genera (*Proteus*, *Morganella*, *Rettgerella*, and *Providencia*) for a group of organisms unique among the Enterobacteriaceae in being able to deaminate phenylalanine (20, 30, 31). So many antigens are shared by members of the different genera that it might be more profitable to regard the family as a genus and reduce the different genera to species level; naturally there would be initial difficulties but ultimately both the taxonomy and nomenclature of the group would be simplified. A start has been made by many workers and Ewing (17) has shown that nearly all *Shigella* serotypes share antigens with the *Escherichia* group.

In attempting to classify bacteria we should bear in mind that only the individual is real (29) and that when we group together several individuals we are creating a unit (species, serotype, etc.) that exists only in our imaginations. To quote Locke again "it is plain that *our distinct species* are *nothing but distinct complex ideas, with distinct names annexed to them*". It is unfortunate that bacteriologists always associate taxonomy and nomenclature, and many of them try to make their classifications agree with ill-informed conceptions of the rules for naming organisms.

The species concept has long taxed the minds of bacteriologists, whose ideas vary from the elastic to the Linnaean, with species fixed and defined at the Creation. An elastic conception seems best to fit known facts, but it is possible that the apparent variability of characters shown by some species may be due more to lack of uniformity in laboratory techniques than to inherent species instability. Clarke (12) appealed for greater standardization of laboratory methods which, she thought, would do more for bacterial characterization than increasing the sensitivity of tests. Discrepant results, usually attributed to the organism, may occur even in the absence of change in the bacterium unless bacteriologists use defined media.

So far I have discussed chaos in relation to taxonomy, but it extends even more widely over the related field of nomenclature. Nomenclature in microbiology has always had a Linnaean tradition, and the different disciplines accept codes or sets of rules which follow more or less the same pattern (4). Drafts of the Bacteriological Code were drawn up early in 1939, but the first Code was not officially adopted until 1947 and published in 1948 (11). Minor revisions were made at congresses in 1950 and 1953 and a revised and annotated edition has been authorized but not yet published.

The first step in creating order in bacteriological nomenclature was the establishment of an International Committee on Bacteriological Nomenclature, of which Dr. R. St. John-Brooks and Dr. R. S. Breed were the first permanent joint secretaries. After the Second World War the Committee was reorganized and greatly enlarged, and now has as chairman Professor E. G. D. Murray. There are several Subcommittees composed of experts to advise on taxonomic problems, and a Judicial Commission which, in addition to issuing opinions on problems of nomenclature, also acts as an executive body for the much larger Nomenclature Committee.

The first problem of nomenclature must be the choice of a starting date and the First International Congress for Microbiology fixed 1753, the publication date of Linnaeus's *Species Plantarum*. In my opinion this was unwise, because it forced attention on names used even before the days of Pasteur, when descriptions were, in the modern sense, nothing short of the farcical. *Serratia* is an excellent example; the name was devised by Bizio to perpetuate the name of the inventor of the steamboat, and the description (in translation (26)) reads "Small, stemless fungi; hemispherical capsules occurring in clusters. *S. marcescens*. A very thin vesicle filled at first with a pink, then with a red fluid". Such a description might apply to any living object that produces a pink or red pigment, and since 'bleeding polenta' can be produced experimentally either by a yeast or by a bacterium, the 'hemispherical capsules' are more likely to refer to a yeast than to a bacterium. At the time of Bizio's work the resolving power of microscopes might have been adequate to show the larger yeasts; it was certainly inadequate to reveal the small rod, first named *Monas prodigiosa*, whose description by Ehrenberg in 1849 was as fantastic as that of Bizio. It is quite impossible to recognize bacteria from these early descriptions and the recommendation of Winslow and his colleagues (35) that the starting date should be 1883 is more realistic than the action of the International Congress. Even 1883 is probably too early, for the era of pure cultures began later in the nineteenth century, and for the anaerobes awaited the development of the McIntosh and Fildes jar during the 1914–18 war.

The development of freeze-drying methods for preserving bacteria has made possible the successful application of the type concept to bacteriology; by this means a name (or at least the specific epithet) is attached to a culture whose description can be confirmed by examination of a preserved culture. In the Bacteriological Code the type concept has been greatly strengthened by the revision of Rule 9 (21), and at its next meeting the Nomenclature Committee may expect to have before it recommendations for the selection of several neotypes, i.e. types designated subsequently in the absence of a designation by the original author (e.g. *Escherichia coli*, *Staphylococcus aureus*, and many *Shigella* serotypes). As neotypes are designated by the Nomenclature Committee, a degree of permanence would be added to the name if, at the same time, the specific epithet could be conserved against earlier names. This action would not guarantee permanence, for with change of genus, a

species might become a later homonym of a species in the new genus. For example a newly described *Nocardia alba* might later be transferred to the genus *Streptomyces*, when a change of epithet would become imperative unless the species were identical with *S. albus* (Rossi Doria) Waksman and Henrici.

A logical development of the type concept is the withholding of recognition from species for which a type (including neotype) culture is not available; such a move would immediately remove, as sources of confusion, the names of hundreds of species with descriptions, or lack of descriptions, so inadequate that they are not now recognizable. It would also automatically remove those bacteria which had not been isolated in pure culture or could not be maintained in the laboratory (e.g. the leprosy bacillus); however the number of such organisms is continually decreasing, and even cultures of leptospira have recently been successfully freeze-dried (6).

Another means of preventing further additions to existing chaos is the suggestion of Ainsworth and Ciferri (3) for the registration of new names; applied to bacteria the Code could require that new names and new combinations should be registered and published in an official organ of the Nomenclature Committee, either with a full description or with a reference to a description published elsewhere; in addition the place of deposition of the type specimen should be recorded.

The Bacteriological Code has followed, some will think too slavishly, the Botanical Code, and exception is often expressed to Rule 23 which, as amended in 1953 says "A legitimate name or epithet must not be rejected merely because it is inappropriate, or disagreeable, or because another is preferable or better known, or because it has lost its original meaning" (22). Bacteriologists strongly object to abandoning a well-known name for an older one discovered perhaps in an obscure journal, but it is difficult to see how the principle of priority, itself intended to avoid creating chaos, could be abandoned to allow well-established names to survive. However, nomenclature is intended to be of service to the taxonomist and not his master (2) and, unlike the botanists, who in general seem to be opposed to official names (18, 33, 34), bacteriologists may attain nomenclatural stability by the creation of lists of conserved specific epithets, many of which, sympathetically considered by the Judical Commission and the Nomenclature Committee, might be exceptions to the rules. For example the following combinations are correct according to the rules but they have probably never been used and are unlikely to find favor: *Escherichia escherichii* (for *E. coli*), *Aerobacter aceticum* (for *A. aerogenes*), *Klebsiella pneumoniae-crouposae* (for *K. pneumoniae*) (13).

Bacteria are difficult to classify because they have not yet heard of the Bergey Manual; this is a paraphrase of a statement made by a well-known American bacteriologist, and while the bacteria remain in such blissful ignorance, we, in our innocence, will continue to try to put them in pigeonholes, on punchcards, or other devices for their orderly management. But shall we ever succeed in making them behave as we would wish, that is free from those intermediate forms so inconvenient to the taxonomist?

For order to come to bacterial systematics all workers must do some plain thinking and most of them will have to abandon at least one cherished idea, name, method, or scheme. First things should come first, and a determined attempt should be made to standardize technical methods used in characterization, not with the idea of calling a halt to technical advances, but in an attempt to achieve a uniformity in quality of descriptions. More use should be made of defined media, and when this is impossible, details should be included of the make or brand of reagents, e.g. peptone is a name attached to a variety of products, but Bacto-Casamino Acids, Bacto-Peptone, Bacto-Tryptone, Bacto-Tryptose, Bacto-Protone indicate products of which something at least is known of the chemical properties of typical batches (15). New species should be adequately studied and, before they are described, compared with known related species: published descriptions should be full and should state methods, findings, comparisons, and the proposed allocation to a genus. A type specimen should be named and made available to other workers. Old species need restudy, preferably by groups of experts using standardized methods. To avoid scattering these descriptions, a limited number of journals might be recognized as official, and a brief summary should be published in a regular publication of the Nomenclature Committee. Neotype strains should be selected and, when approved, deposited in culture collections. New names should be controlled by the Register of Names (3), and lists of conserved names prepared and approved as different groups of bacteria are studied and written up as monographs.

These, then, are some of the steps we must take to bring a semblance of order from bacterial chaos; but when we have taken them we shall not have answered all the questions. With so much regimentation and officialdom, will the bacterium's life be worth living, and will it, at last, bow to man's will and remain docile and unchanging long enough to justify the fulfillment of man's perpetual wish to stick a label on it?

What, too, will become of the bacteriologist who rebels against publication in official or approved journals, and against registration of the name or names he bestows on his test-tube offspring? Will he, like the indolent parent who fails to register the birth of his child, be fined forty shillings for each offence (7), or will he merely be applauded by the rest of his fellow workers as one of Jo'burg's bacterial adventurers (28), a survivor of a glorious past when men were men and bacteria were "bugs".

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ANTIGENIC RELATIONSHIPS BETWEEN LISTERIA MONOCYTOGENES AND STAPHYLOCOCCUS AUREUS[1]

By H. P. R. Seeliger and F. Sulzbacher

Abstract

The antigenic relationships between the different serotypes of *Listeria monocytogenes* and *Staphylococcus aureus* were investigated by means of agglutination, agglutinin-absorption, precipitation, and complement fixation tests. Reciprocal cross-reactions were demonstrated with serotypes 1, 2, and 3 of *L. monocytogenes* and many *S. aureus* strains, but not with serotypes 4*a* and 4*b*, using O suspensions of *L. monocytogenes* and autoclaved suspensions of *S. aureus*. Soluble heat-stable polysaccharide extracts, on the contrary, were quite specific in precipitin tests. Antisera against *L. monocytogenes* type 3 cross-precipitated unilaterally extracts of most *S. aureus* strains. In the absence of clear-cut serological species specificity in these two microorganisms, in their reactions with rabbit antisera, and with human sera from suspected cases of listeriosis, there is need for caution in the interpretation of positive serological tests as an aid to diagnosis.

Introduction

Since the discovery of listeriosis and the exhaustive description of its etiologic agent by Murray, Webb, and Swann (9) thirty years ago, *Listeria monocytogenes* and its various clinical manifestations in man and animals have become a subject of intensive studies for numerous workers in all parts of the world. A recent monograph (18) lists some 350 references; and the number of publications is rapidly increasing.

Among the various problems of listeriosis research, the serology of the organisms and the antibody response in the infected animal are of special interest. Although *L. monocytogenes* contains a non-antigenic lipid (20) that enhances antibody formation in rabbits immunized with other bacteria—as proved by Stanley (21) and by Girard (4, 5) in Murray's laboratory—the antibody response of infected animals and man is often poor. On the other hand, antibodies to *Listeria* spp. are not uncommonly encountered in low dilutions of sera from apparently uninfected men and animals. The frequency of these agglutinin titers has not yet been satisfactorily explained. There is no bacteriological evidence that the majority of these titers in normal healthy individuals and domestic animals results from *Listeria* infection.

The agglutination of *Listeria* suspensions by horse serum has been described as non-specific and doubt has also been expressed about the specificity of agglutinins in human convalescent serum after treatment of the patient with horse serum. It has also been suggested that heterologous antibodies against antigens other than *L. monocytogenes* may be responsible for the observed titers (18).

Although results of earlier investigations do not support the latter hypothesis, in recent years several examples of reciprocal and unilateral cross-reactions between *L. monocytogenes* and other bacteria have been described.

[1]*Manuscript received October 29, 1955.*
Communication from the Hygiene-Institut der Rheinischen Friedrich Wilhelms-Universitat Bonn/Germany. Director: Prof. Dr. Dr. H. Eyer.

For instance it was found that antiserum against *Escherichia coli* K8 (08) precipitated the non-antigenic *Listeria* lipoid substance (7). Seeliger reported antigenic relationships between *L. monocytogenes* and enterococcus strains of the *Streptococcus fecalis* group, and drew attention to the importance of this observation with regard to the interpretation of serum agglutinins against both species (16). The observations of Drew (2) and our own observations raise the question whether *Staphylococcus* antigens might play a similar part in the serology of listeriosis.

A search was therefore made for antigens common to the serotypes of *L. monocytogenes* and staphylococci. The following report mainly concerns results with rabbit antisera. Some results with human sera from healthy persons and from patients suspected to have listeriosis are included. The experimental investigations were in part performed by Miss Beeck, the details of whose work, including experiments with sera from patients with *S. aureus* infections, will be published separately (1).

Materials and Methods

Strains

The strains used are listed in Table I. They were kept in nutrient agar stabs at room temperature and were tested for purity and typical cultural and biochemical reactions by appropriate methods before use.

TABLE I

LIST OF CULTURES

Strain	Designation	Source
L. monocytogenes, serotype 1	7973*	H.I.B.†
L. monocytogenes, serotype 2	5348*	H.I.B.
L. monocytogenes, serotype 3	5105*	H.I.B.
L. monocytogenes, serotype 3	1702/54*	Sheep brain‡
L. monocytogenes, serotype 4*a*	5214*	H.I.B.
L. monocytogenes, serotype 4*b*	1071/53*	Spinal fluid, fatal meningitis in man§
S. aureus	B1 to B7 (B2* and B3*) B8 to B20	H.I.B. Pus, wounds, abscesses, bile, and pathological fluids in man‖
S. aureus, eight serotypes	2095/49 3647/49 1503/49 28/51 2253/51 17A F21 S365	Dr. Oeding, Bergen

*Strains used for the production of antisera.
†Culture collection of the Hygiene-Institut, Bonn.
‡Isolated by Dr. Linsert, Greifswald: typed by Seeliger.
§Isolated by Dr. Preuss, Wuppertal; typed by Seeliger.
‖Isolated at the clinical laboratory of the Hygiene-Institut, Bonn.

Antigens

Several preparations of whole cell antigens were made:

(*a*) living organisms from nutrient agar slants grown for 18 hr. at 37° C.;

(*b*) formalinized saline suspensions of *S. aureus* strains B1 to B20 grown on nutrient agar for 18 hr. at 37° C.;

(*c*) saline suspensions of all the *S. aureus* strains after 18 hr. growth on nutrient agar, which were autoclaved for one hour at 121° C.;

(*d*) O suspensions of all the *L. monocytogenes* and *S. aureus* strains, after they were heated for one hour at 100° C.

The *L. monocytogenes* cultures were grown for 24 hr. at 37° C. on buffered 1% dextrose peptone agar, pH 7.2. Owing to spontaneous agglutination of the boiled suspensions to which 0.5% phenol was added, the *Listeria* O suspensions were treated in the ultrasonator for 5–10 min. with a frequency of 1 mHz. at 32–38 w. Since most of these antigens were slightly acid, all suspensions were carefully neutralized with sodium hydroxide, in order to exclude any acid agglutination. *L. monocytogenes* suspensions prepared by this method (14) are salt-stable and in the refrigerator remain so for at least six months.

The *L. monocytogenes* O suspensions were used for the production of antisera as well as for agglutination, agglutinin-absorption, and complement fixation tests (CFT). The formalinized *S. aureus* suspensions were used for preliminary agglutination tests; most of the serologic tests were made with the autoclaved *S. aureus* suspensions.

Soluble polysaccharides for precipitin tests were prepared as follows. The growth of *S. aureus* on nutrient agar in Drigalski dishes was harvested after 24 hr. at 37° C., and the growth of *L. monocytogenes* on buffered 1% dextrose peptone or tryptose agar after 24–48 hr. at 37° C. The growth from each dish was suspended in 10 ml. isotonic phosphate-buffered saline, washed once, and boiled for 60 min. The boiled suspensions were centrifuged for 30 min. at approximately 3000 r.p.m. and the sediment was discarded. To the clear supernatant four volumes of 96% ethyl alcohol were added, and the mixture was allowed to stand at 4° C. for 18–24 hr. The resulting white precipitate was spun down and dissolved in 5 ml. phenolized phosphate-buffered saline. This extract, which reacted strongly positive in the Molisch test, was used without further dilution.

Antisera

Rabbit antisera were obtained after serial intravenous injection of increasing amounts of *L. monocytogenes* O suspensions and of formalinized *S. aureus* suspensions (see 19, 18) made from the strains in Table I marked with an asterisk.

When sufficiently high agglutinin and precipitin titers had developed, the rabbits were bled by cardiac puncture. The sera were inactivated for 30 min. at 56° C. A portion of each was stored in the frozen state and used for precipitin and complement fixation tests; the remainder was preserved by the

addition of an equal volume of pure glycerol and used in agglutination tests. The pre-immunization normal serum of each animal was stored in the frozen state for use as control in tests with the antisera.

Human sera from normal persons and from patients with suspected *Listeria* infection were received from various hospitals and tested after inactivation for 30 min. at 56° C. Hemolyzed or turbid specimens were rejected.

Test Procedures

The tests were: slide- and tube-agglutination test (19, 18); agglutinin-absorption test (19); precipitin test in tubes (2, 19) and in agar-gel (17); CFT with standardized antigens (14, 18). All dilutions and titrations in the agglutination, absorption, and precipitin tests were made with phosphate-buffered 0.85% saline, pH 7.2. In the CFT veronal buffer solution was used. Most titrations were repeated twice, and many experiments thrice. Agglutination titers were determined by the aid of an agglutinoscope; and both these and CF titers are throughout the paper expressed as reciprocals of the serum dilution.

Results

A. Rabbit Sera

The relative frequency of staphylococcal agglutination by rabbit immune antisera against *L. monocytogenes* was determined in slide agglutination tests, using 1:5 antiserum and living, formalinized, boiled, and autoclaved suspensions of 20 *S. aureus* strains.

Although a number of live *S. aureus* cultures was strongly agglutinated by one or more *L. monocytogenes* antisera, about 50% of the strains failed to agglutinate. Most of the boiled and formalinized suspensions did not react with any of the *Listeria* antisera. A high percentage of the autoclaved suspensions, however, were agglutinated by type 1, 2, and 3 *L. monocytogenes* antisera, but not by 4*a* and 4*b* antisera. Tube agglutination tests yielded titers up to 1280 in type 1 antiserum (homologous titer 5120). *S. aureus* strains B2 and B3 reacted with *L. monocytogenes* type 3 antiserum to a higher titer (10,240) than the homologous titer (5120) (1).

By comparison of the titers in two rabbits immunized with *L. monocytogenes* type 3 before, during, and after immunization, it was found (1) that the titers with *S. aureus* B2, B3, and 17A paralleled the homologous *Listeria* titers. In the same way, a definite rise of *L. monocytogenes* type 1 and 3 titers occurred during immunization of rabbits with formalinized suspensions of the two cross-reacting *S. aureus* strains B2 and B3. After absorption of type 1 *L. monocytogenes* O antiserum with autoclaved *S. aureus* B3, the staphylococcal agglutinins disappeared, but the homologous O titer was not altered. On the other hand, absorption of *S. aureus* B3 antiserum with O suspension of *L. monocytogenes* type 1 reduced the homologous titer from 2560 to 640. Absorption of this antiserum with O suspensions of both *L. monocytogenes* type 1 and type 3 or with type 3 suspension alone, reduced the titer to 160. Cross

TABLE II

TUBE AGGLUTINATION AND ABSORPTION TESTS WITH ANTIGENS AND ANTISERA OF *L. monocytogenes* AND *S. aureus*

Serum	Absorbed with	Homologous titers	Autoclaved suspensions		
			S. aureus B2	B3	17A
L. monocytogenes type 1	Nil	5120	1280	1280	640
	S. aureus B3	5120	—	—	—
Normal serum 78	Nil	80	80	80	40
Serum 78 after immunization with *L. monocytogenes* type 3(5105)	Nil	5120	10240	10240	10240
	S. aureus B3	640	—	—	—
Normal serum 79	Nil	80	80	80	80
Serum 79 after immunization with *L. monocytogenes* type 3(1702)	Nil	10240	10240	10240	10240
	S. aureus B2	160	—	—	—
L. monocytogenes type 4*a*	Nil	2560	—	—	—
L. monocytogenes type 4*b*	Nil	10240	80	80	80

			O suspensions of *L. monocytogenes*				
			Type 1	Type 3(5105)	Type 3(1702)	Type 4*a*	Type 4*b*
Normal serum 77	Nil	80	40	40	40	20	20
	S. aureus B3	—	40	—	—	20	—
Serum 77 after immunization with *S. aureus* B3	Nil	2560	640	640	1280	40	20
	L. monocytogenes type 3	160	—	—	—	20	—
	L. monocytogenes type 1	640	—	160	n.d.	n.d.	—
	L. monocytogenes types 1 and 3	160	—	—	—	20	—
Normal serum 82	Nil	80	40	40	40	20	20
Serum 82 after immunization with *S. aureus* B2	Nil	2560	320	1280	1280	40	20
	L. monocytogenes type 3	640	40	—	—	40	—

n.d.—not done.

absorption with antigens and antisera of *S. aureus* B2 and B3 and two strains of *L. monocytogenes* type 3 resulted in a considerable decrease of homologous titers in each instance.

These results, summarized in Table II, are strong evidence of major antigenic relationships between *L. monocytogenes* type 3 and *S. aureus* strains. There are minor relationships between *L. monocytogenes* type 1 and staphylococci. Since the O antigens of type 1 and 2 of *L. monocytogenes* are identical, the reactions of autoclaved *S. aureus* with *L. monocytogenes* type 2 antisera, as observed by slide agglutination, can be explained on the same basis.

Precipitin tests with undiluted sera and polysaccharide extracts confirmed only in part the results with agglutination and agglutinin-absorption experiments. There were no visible cross-reactions between the polysaccharides of *L. monocytogenes* types 1, 2, 4*a*, and 4*b* and staphylococcal antisera, or between staphylococcal polysaccharides and the corresponding types 1, 2, 4*a*, and 4*b* antisera. But there were cross-reactions between *L. monocytogenes* type 3

TABLE III

PRECIPITIN TESTS WITH ANTISERA AND POLYSACCHARIDE EXTRACTS OF *L. monocytogenes* AND *S. aureus*

Extract	Antiserum					
	L. monocytogenes type				*S. aureus*	Normal serum
	1	3 (5105)	3 (1702)	4*b*	B3	
L. monocytogenes type 1	+++	+++	+++	−	−	−
L. monocytogenes type 3 (5105)	−	+++	+++	−	−	−
L. monocytogenes type 3 (1702)	−	+++	+++	−	−	−
L. monocytogenes type 4*b*	−	−	−	++	−	−
S. aureus B1	−	+	++	−	++	−
S. aureus B2	−	+++	++	−	++	−
S. aureus B3	−	+++	+++	−	+++	−
S. aureus B4	−	++	++	−	+	−
S. aureus B5	−	++	+++	−	++	−
S. aureus B6	−	+++	+++	−	+	−
S. aureus B7	−	+++	+++	−	+++	−
S. aureus B8	−	+++	++	−	+++	−
S. aureus B9	−	+++	+++	−	++	−
S. aureus B10	−	++	+++	−	+++	−
S. aureus B12	−	+++	+++	−	+++	−
S. aureus B14	−	+++	+++	−	+++	−
S. aureus 17A	−	+++	+++	−	+	−
S. aureus F21	−	++	+	−	+++	−
S. aureus 3189/51	−	++	+	−	+++	−

antisera and *S. aureus* extracts. This was a purely unilateral cross-reaction since polysaccharide antigens of *L. monocytogenes* type 3 consistently failed to precipitate such *S. aureus* antisera prepared against cross-reacting strains (Table III).

By the application of the agar-gel technique the cross-reacting systems could be seen as precipitation bands. In accord with the outcome of the tube tests, cross-reactions occurred with both the *L. monocytogenes* type 3 antisera and staphylococcal polysaccharides, but not with *S. aureus* B2 and B3 antisera and extracts of *L. monocytogenes* types 1, 2, 3, 4*a*, and 4*b*. In Fig. 1, for example, polysaccharide extracts of three different *S. aureus* strains, including Oeding's type strain 17A, have produced identical precipitation bands with *L. monocytogenes* type 3 antiserum. The identity is indicated by arcade formation. Although with the homologous type 3 extract three separate bands develop, all three unite with the staphylococcal precipitation bands; from this, a close similarity of the reactive carbohydrates can be deduced.

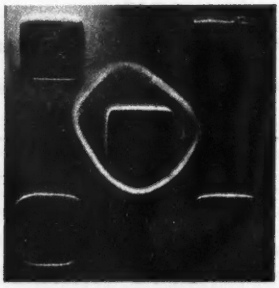

FIG. 1. Agar-gel precipitation. *L. monocytogenes* antiserum type 3 in the central reservoir; and polysaccharide extracts of *L. monocytogenes* type 3 in left upper, *S. aureus* B2 in right upper, *S. aureus* B3 in left lower, and *S. aureus* 17A in right lower reservoir.

The failure to obtain precipitation of *L. monocytogenes* type 3 extracts by the *S. aureus* antisera was not due to the absence of strong antibodies, because the antisera contained precipitins for extracts of *S. aureus*; moreover, cross-reacting antisera were not produced, even after prolonged immunization.

Pronounced reciprocal cross-reactions occurred, however, in complement fixation tests, summarized in Table IV. The uniformly negative results with the pre-immunization control sera have been omitted from this Table. The results of the CFT agree with those of the agglutination tests. This is to be expected because in both agglutination and CF tests, whole cells were used as antigens. When the agglutination and CF reactions are compared with the precipitin reactions, it is clear that parts of the antigenic complexes other than the extractable carbohydrate material are responsible for the observed cross-reactions.

TABLE IV

THE COMPLEMENT FIXATION REACTIONS OF ANTISERA AGAINST *L. monocytogenes*, TYPES 1, 3, AND 4*b* AND *S. aureus* B2 AND B3 WITH HOMOLOGOUS AND HETEROLOGOUS ANTIGENS

	Antisera					
	L. monocytogenes type				*S. aureus*	
Antigen	1	3 (5105)	3 (1702)	4*b*	B2	B3
L. monocytogenes, type 1	444442				443000	444400
L. monocytogenes, type 3 (5105)		444444	444444		444300	444100
L. monocytogenes, type 3 (1702)		444444	444444		444000	444300
L. monocytogenes, type 4*b*				444320	000000	200000
S. aureus B2	444100	444432	444441	440000	444300	444400
S. aureus B3	444400	444400	444443	420000	442000	444431
All antigen and serum controls: 0						

**Percentage of inhibition of hemolysis in twofold dilution series from 1:20 to 1:640. 4, 3, 2, 1 = 100, 75, 50, 25% inhibition of hemolysis.*

TABLE V

REACTIONS OF HUMAN SERA WITH O SUSPENSIONS OF *L. monocytogenes* TYPES 1 AND 3 AND AUTOCLAVED SUSPENSIONS OF *S. aureus* STRAINS IN AGGLUTINATION AND AGGLUTININ-ABSORPTION TESTS

Human serum	Absorbed with *S. aureus*	Agglutination with *L. monocytogenes* 0-1	*L. monocytogenes* 0-3	*S. aureus* B2	*S. aureus* B3	*S. aureus* B5	*S. aureus* B12	*S. aureus* B15
C 1329/54		640	640	80	1280	320	1280	1280
	B2	320	320	—	—	—	—	—
	B3	320	320	—	—	—	—	—
C 141/55		320	640	20	640	320	320	160
	B3	160	320	—	—	—	—	—
C 84/55		160	40	—	640	40	640	—
	B5	80	20	—	20	—	—	—
C 85/55		80	640*	—	40	40	40	—
	B12	80	20	—	—	—	—	—
C 626/55		320	320		640			
	B3	80	—		—			
C 1319/55		320	80		160			
	B3	320	—		—			
C 1321/55		40	80		160			
	B3	20	—		—			
C 1339/55		1280	320		1280			
	B3	1280	—		—			
C 1341/55		40	40		320			
	B3	20	—		—			

**Partial agglutination.*
— = negative at 1:20.

B. Human Sera

Cross-agglutination of *S. aureus* strains by human sera containing agglutinins for O suspensions of types 1 or 3 was observed almost regularly (Table V). In several sera the *S. aureus* titers exceeded the *Listeria* titers by two- or four-fold; as in serum C 1329/54, from a child suspected of suffering from listeriosis. The maximum *S. aureus* agglutinin titer observed was 1280.

As Table V shows, absorption of these sera with autoclaved suspension of various *S. aureus* strains as a rule failed to reduce the O agglutinins for *L. monocytogenes* type 1 more than twofold. In most instances, O agglutinins for type 3 were greatly reduced, sometimes to nil.

The pattern of CFT performed with human sera was different. Positive (+ to ++++) reactions in serum dilutions of 1:5 or higher were obtained with the following frequencies in 807 sera.

L. monocytogenes type 1	61
Associated with *S. aureus* B3	4
L. monocytogenes type 3	125
Associated with *S. aureus* B3	43
L. monocytogenes type 1 and type 3	72
Associated with *S. aureus* B3	25
S. aureus B3	47

TABLE VI

EFFECT OF ABSORPTION WITH *S. aureus* ANTIGEN IN QUANTITATIVE COMPLEMENT FIXATION TESTS WITH HUMAN SERA

Serum	Absorbed with	Titer with *L. monocytogenes* type 1	Titer with *L. monocytogenes* type 3	Titer with *L. monocytogenes* type 4b	Titer with *S. aureus*	Serum control
C 622/55	Nil	5+++ 10++	5+++	—	5++++	—
	S. aureus B3	5+++	5+++	—	—	—
C 940/55	Nil	10++++ 40++	5+++ 10++	5+++	5+++	—
	S. aureus B3	10++++ 20++	5+++	5+++	—	—
C 2235/55	Nil	40+++	10+++	5++++	10+++	
	S. aureus B3	10++++ 40++	—	—	—	—
C 2197/55	Nil	10++ 20±	5+++ 10++	—	40+++	—
	S. aureus B3	10++	5±	—	—	—
Antigen control		—	—	—	—	

++++, +++, ++, *and* + = *100, 75, 50, and 25% haemolysis.*

It is quite obvious that the highest frequency of CF cross-reactions occurred in human sera reacting with *L. monocytogenes* type 3 antigen. The *L. monocytogenes* titers of type 1 usually exceeded the *S. aureus* titers.

By absorption it was possible to exhaust the *S. aureus* titers separately without a simultaneous disappearance of the CF titer with *L. monocytogenes* type 1. This is shown in Table VI. When several sera positive by agglutination and CF tests for *L. monocytogenes* type 1 were absorbed with O suspensions of *L. monocytogenes* type 1, all antibodies against *Listeria* and *S. aureus* antigens disappeared.

Discussion

Serologic cross-reactions between antigens of entirely different families, genera, and species of bacteria have been observed by numerous investigators. Similar relations exist between intestinal bacteria and blood group substances (6), *Sporotrichum schenckii* and pneumococci (10) or *Geotrichum candidum*, *Endomyces magnusii*, and *Nadsonia elongata* (15). Antisera against the capsular substances of *Cryptococcus neoformans* precipitate polysaccharides from many other sources (3).

The number of antigenic substances is clearly not unlimited and serologic cross-reactions between quite different organisms might be found with some regularity if a systematic search were made. It must also be remembered that serologic cross-reactions are not confined to identical antigens or part of them. Some similarities in the chemical structure may be sufficient to determine a serologic overlapping. The overlap, moreover, may be reciprocal or unilateral; for instance, the cross between *G. candidum* and *N. elongata* is reciprocal, but both species are related only unilaterally to *Lipomyces lipoferus* (15).

Absolute specificity cannot be expected from serologic reactions. In many instances the specificity is only relative. This is true of the *Listeria* spp., as shown by the relation between *L. monocytogenes* and *S. fecalis* strains (16) and by reciprocal and unilateral relationships we have demonstrated between antigens of *Listeria* and *S. aureus*, although the organisms belong to entirely different families.

Owing to the complexity of the *S. aureus* antigens (11, 12, 13), no attempt was made to identify the antigens or partial factors common to the majority of *L. monocytogenes* and *S. aureus* serotypes.

There are pronounced differences among the *L. monocytogenes* serotypes with regard to their antigenic relationships to staphylococci. Whereas serotypes 4*a* and 4*b* were serologically quite different from all the *S. aureus* strains tested, reciprocal cross-reactions occurred between serotypes 1, 2, and 3 of *L. monocytogenes* on the one hand and several *S. aureus* serotypes on the other, both in agglutination and complement fixation tests. Possibly the antigenic fraction II common to these *L. monocytogenes* types is involved in these cross-reactions, since absorbed sera containing antibodies for fraction I only did not agglutinate *S. aureus*.

The precipitin reactions of polysaccharide extracts were highly type- or species-specific reactions, except with antisera against *L. monocytogenes* serotype 3, which precipitated extracts of types 1, 2, and 3 and of most of the *S. aureus* strains as well. This confirms Drew's (2) work with partially purified polysaccharide extracts made by Fuller's method. The reaction was purely unilateral since type 3 extracts did not react with *L. monocytogenes* type 1 and 2, or with *S. aureus*.

In his studies with *S. aureus* antisera, Oeding (12, 13) observed that *Staphylococcus* antigens prepared in different ways differed in their reaction pattern. The same is true of reactions with *L. monocytogenes* antisera. Autoclaved suspensions, although salt-stable, had a significantly wider reactivity than formalinized or boiled antigens. The agglutinability, antigenicity, and agglutinin-binding properties also varied among several strains tested. For instance *S. aureus* B2 was agglutinated only poorly by some human sera, but it was able to absorb *S. aureus* agglutinins even for strains which were agglutinated by much higher serum dilutions. Several highly agglutinable strains absorbed poorly.

The cross-reactions between *Listeria* and staphylococci were not limited to the autoclaved antigens. Between 30 and 50% of the *S. aureus* strains were agglutinated by one or more *L. monocytogenes* antisera, when living suspensions were tested. This may be due to labile surface antigens. In this connection it is noteworthy that *S. aureus* and *L. monocytogenes* toxins appear to be related; Girard (4, 5) notes that "the *L. monocytogenes* toxin preparation seemed to be neutralized by standard *S. aureus* alpha anti-toxoid, indicating a possible relationship between the two".

The practical implications of these cross-reactions for the serodiagnosis of listeriosis are obvious. At the present time only little is known of the

specificity of the frequent "*Listeria*" titers in man and animals. It is remarkable that in Germany such titers are usually observed against type 1 and 3 of *L. monocytogenes* only, not against type 4*a* and 4*b*. Type 1 is known to be prevalent and the other types are relatively rare in Germany. In the CFT, positive reactions with type 3 antigen are frequent, but they cannot be interpreted as a consequence of type 3 infections, because type 3 organisms seem to be extremely rare. One may speculate that O titers for type 1 also are not specific for *Listeria* because of the antigenic relationships of this organism with enterococci and staphylococci. Several observations, however, are not consistent with this view. In absorption tests, enterococcus and *S. aureus* antigens usually fail to affect the agglutinin or CF titers for *L. monocytogenes* type 1 in sera from suspected cases of listeriosis. This, of course, does not exclude the possibility that other antigens may be responsible for the "*Listeria*" titers. The assumption that the titers are non-specific is even more unlikely when H-agglutinins against *L. monocytogenes* are found. It would be a strange coincidence if these agglutinins also were due to heterologous antigens; and the association of O- and H-antibodies against *L. monocytogenes* is strong evidence for the specificity of the reaction.

It is well known that serologic cross-reactions demonstrable with rabbit immune sera do not necessarily appear in human sera. In agreement with this, it was found that in spite of the cross-reactions of *L. monocytogenes* and *S. aureus* antigens in rabbit sera, positive CF reactions were relatively uncommon with *Listeria*-positive human sera and *S. aureus* antigen.

It would be of great interest to determine whether "*Listeria*"-antibodies appear in the sera of individuals with acute or chronic staphylococcal infections. Lang (8) recently reported that no such antibodies developed in children with proved *S. aureus* infections; but more cases must be studied before any final conclusions can be drawn. Such a study is in progress (1).

In the light of all these findings it appears that the interpretation of positive "*Listeria*" titers is rather difficult, and must be made with extreme caution. Only positive reactions with both standardized O and H antigens, associated with a positive CF reaction, have a reliable diagnostic significance. The agglutination titers should be 320 or more, though lower titers do not exclude *Listeria* infections. Repeated examinations will establish a curve of antibody products in acute infections. Stationary titers in the absence of a case history compatible with listeriosis give no certain indication of *Listeria* infection; but in acute cases a rise and a fall of agglutinin and CF titers are consistent with the diagnosis of infection. By use of these criteria serologic evidence of *Listeria* infection was obtained in 55 cases out of 2023 examined during a period of 22 months.

The application of serologic procedures has its strict limitations in the diagnosis of many infectious diseases, including listeriosis. The absence of critical interpretations of serum titers has more than once brought valuable tests into discredit. The serology of listeriosis should therefore be explored further before the clinical and epidemiological value of serological tests is

established; and such tests should be corroborated by bacteriological findings, the only reliable proof. Every effort should be made to isolate *L. monocytogenes* from clinically and serologically suspected cases.

Acknowledgment

We are greatly indebted to Dr. Per Oeding, Bergen, Norway, for the serotypes of *S. aureus* used in this study.

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THE ENTEROBACTERIAL HEMAGGLUTINATION TEST AND ITS DIAGNOSTIC POTENTIALITIES[1]

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Abstract

Crude *Salmonella* antigens obtained from heated cultures are readily adsorbed on human red blood cells; the latter are specifically agglutinated by homologous bacterial antibodies. This *Salmonella* hemagglutination test is shown to be a sensitive tool for the detection of antibodies developed during salmonellosis and is distinctly superior to the conventional bacterial agglutination test. Patients with *Salmonella* infection may develop homologous hemagglutinins in high titer and a variety of heterologous enteric antibodies in low titer. These antibodies can be differentiated by absorption with modified red blood cells. Three different purified *Escherichia coli* lipopolysaccharides as well as the corresponding crude antigens can be simultaneously adsorbed on red blood cells. A suspension of red blood cells modified with several *Salmonella* and *Shigella* antigens is shown to be suitable as a polyvalent antigen for the detection of enterobacterial antibodies. The possible clinical usefulness of the monovalent and polyvalent enterobacterial hemagglutination tests is discussed.

To Professor E. G. D. Murray
"Le style c'est l'homme." (Voltaire)

Introduction

"Blut ist ein ganz besondrer Saft." (Goethe)

Australian workers were the first to report that a variety of bacterial antigens, polysaccharide in nature, are readily adsorbed by red blood cells and that, although this modification of the erythrocytes does not result in grossly visible effects, such treated cells are agglutinated by homologous bacterial antibodies (6). This indirect, conditioned, or passive hemagglutination test is characterized by extraordinary sensitivity and permits the detection of certain bacterial antibodies when the conventional bacterial agglutination fails to do so. Studies in this laboratory have revealed that this method can be used as a sensitive tool for the demonstration of *Escherichia coli*, *Shigella dysenteriae* (Shiga), and *S. sonnei* antibodies in the sera of immunized rabbits or of patients (9, 10, 11, 12, 13, 18). It appeared reasonable to assume that this hemagglutination method lends itself also to the detection of the antibody response of patients suffering from *Salmonella* infections. The results of this study are embodied in the present report.

The bacterial hemagglutination technique offers another advantage over the bacterial agglutination test aside from sensitivity, namely, it makes possible the use of suspensions of appropriately modified erythrocytes as polyvalent diagnostic antigens for the detection of homologous bacterial antibodies.

[1]*Manuscript received July 20, 1955.*
Contribution from the Laboratory of Bacteriology and the Department of Pediatrics, Children's Hospital, Buffalo, New York; the Departments of Bacteriology and Pediatrics, University of Buffalo, Buffalo, New York; and the Wander Forschungsinstitut, Säckingen, Germany. This investigation was supported by a research grant (E-658) from the National Microbiological Institute of the National Institutes of Health, Public Health Service, U.S.A.

Observations regarding basic features and diagnostic potentialities of such polyvalent enterobacterial hemagglutination tests also are presented in this communication.

Material and Methods

The *Salmonella* hemagglutination test was carried out according to the techniques developed for enteropathogenic *Escherichia coli* (9, 10). Briefly, the *Salmonella* strains, freshly isolated from patients, were grown on brain veal agar in Kolle flasks at 37° C. for 18 hr. The growth was harvested in 25 ml. of physiological saline solution. This suspension was heated in boiling water for one hour, since it was shown that heating releases antigen from the bacterial cells and causes a change of the soluble antigen which renders it more suitable for adsorption on red blood cells (12, 13, 16). The heated suspensions were centrifuged in a refrigerated centrifuge, and the supernates were used in a dilution of 1:10 in the hemagglutination test.

Purified *Escherichia coli* lipopolysaccharides were prepared according to the methods of Westphal and associates (26, 27). The lipopolysaccharides were dissolved in phosphate buffer (pH 7.3) and heated in boiling water for one hour, in order to enhance their erythrocyte modifying capacity (16).

Red blood cells were obtained from healthy blood donors of blood group O and from sheep. A 2.5% suspension was washed thrice in phosphate buffer solution, in order to remove plasma, which interferes with adsorption of enterobacterial antigens by erythrocytes (14, 17, 19). To the sediment of this suspension was added the bacterial antigen; the mixture was kept in the water bath at 37° C. for 30 min. The red blood cells were again washed three times, in order to remove excess antigen.

The hemagglutination test itself was carried out as follows. Inactivated serum from patients (volume 0.25 ml.) in serial dilutions was mixed with modified red blood cells. The mixtures were incubated in a water bath at 37° C. for 30 min. and then centrifuged at 1000 r.p.m. for one minute. The latter procedure markedly enhances the resulting hemagglutination. It may be noted that in a strongly positive test all red blood cells are present in a single clump.

The hemolytic modification of the above hemagglutination test was done as described above except that guinea pig complement (dilution 1 : 20) was added and hemolysis read after incubation in a water bath at 37° C. for 30 min.

The bacterial agglutination test was carried out as follows. Suspensions of the patients' strains were prepared as described above; they were heated in boiling water for one hour. To 0.25 ml. of the suspension was added an equal amount of inactivated patients' serum in serial dilutions. The mixtures were kept in a water bath at 50° C. for 18 hr., and the resulting bacterial agglutination was read grossly.

In the absorption experiments the sediment of appropriately modified red blood cells was mixed with the patient's serum in a suitable dilution. The

mixture was kept in the water bath at 37° C. for 15 min. and then centrifuged. The serum thus obtained was absorbed a second time. For control purposes an aliquot of the serum was treated in identical fashion with nonmodified erythrocytes. The presence of hemagglutinins was then determined as described above.

Sera were obtained from patients with *Salmonella* infections at various stages of the illness. The sera were removed from the blood clots within 24 hr. after the specimens had been procured and kept in the frozen state until used. It must be stressed that paired serum specimens were always tested simultaneously.

As diluent phosphate buffer (Difco) (pH 7.3) was used throughout the experiments.

Results

The Salmonella Hemagglutinin Response of Patients with Salmonella Infections

The antibody response of 15 patients suffering from *Salmonella* infection was determined comparatively by means of the hemagglutination and bacterial agglutination techniques. The results and other pertinent data are presented in Table I.

It can be seen that a fourfold or greater increase in homologous *Salmonella* hemagglutinins was demonstrated in four patients and that high titers (1 : 2560 and up) of these antibodies were encountered in four additional individuals. It is also evident that bacterial agglutinins were either not demonstrated or were found only in low titers. The clinical manifestations of the infection in these patients included diarrheal disease, food poisoning, and sepsis, and a variety of *Salmonella* types were encountered. Demonstrable antibody rises occurred between 8 and 11 days after onset of the illness.

Of the remaining seven patients, three showed *Salmonella* hemagglutinins in a titer of 1 : 160; in one of these patients the first blood specimen was obtained 10 days after onset of the disease, at a time when the rise in antibody titer may have reached its peak; in another only one blood specimen was available. It is interesting to note that of the other four patients, whose hemagglutinin titers did not exceed 1 : 40, three were infants less than one year of age, and in one of them blood specimens were not available more than three days after onset of the illness.

It was previously shown that with immune antisera obtained from rabbits the hemolysin titers were considerably higher than the hemagglutinin titers. For this reason a number of comparative tests were carried out with serum specimens obtained from patients. The results of a typical experiment are presented in Table II.

It is evident that the serum obtained from a patient with *Salmonella* infection up to a dilution of 1 : 1600 produced agglutination of appropriately modified red blood cells of both man and sheep. However, the titer of the corresponding hemolysin was only 1 : 100 with sheep blood cells and less than 1 : 25 with human blood cells. It is obvious therefore that the hemolytic

TABLE I

THE HEMAGGLUTININ RESPONSE OF PATIENTS WITH SALMONELLOSIS

Patient	Age	Disease and onset	*Salmonella* type	Date of blood specimen	*Salmonella* hemagglutinin titer	*Salmonella* agglutinin titer
D. C.	11 yr.	Diarrhea 2–20–54	Oranienburg	2–25–54	20*	—**
				3– 3–54	160	—
				3–13–54	80	—
				3–20–54	80	—
C. M.	3 yr.	Enterocolitis 9–30–54	Montevideo	10– 4–54	20	—
				10– 5–54	40	—
				10– 8–54	1280	10
				10–19–54	1280	10
J. R.	6 mos.	Enterocolitis 10–25–54	Manhattan	11– 1–54	40	—
				11– 5–54	160	—
				11– 8–54	80	—
				11–23–54	80	—
B. M.	Adult	Food poisoning 3–20–55	Muenchen-oregon	3–23–55	80	—
				3–29–55	6400	—
G. B.	3 yr.	Diarrhea 4–9–55	Tennessee	4–25–55	5120	10
				5– 3–55	640	10
M. W.	12 yr.	Diarrhea (?)	Oranienburg	5– 9–55	1280	10
				5–17–55	2560	N.d.***

TABLE I—*Concluded*

J. T.	22 mos.	Sepsis, diarrhea 1–14–55 (?)	choleraesuis	1–25–55	5120	160
				2– 4–55	5120	640
				2–16–55	5120	320
P. F.	12 yr.	Pseudo-appendicitis 4–30–55	Schottmuelleri	5– 6–55	2560	80
				5–11–55	2560	80
D. M.	4 yr.	Diarrhea 1–14–55	Montevideo (and *S. sonnei*)	1–15–54	80	—
				1–20–54	80	—
				2– 9–54	160	—
E. C.	3 yr.	Diarrhea (2–17–54)	Oranienburg	2–19–54	20	—
				2–20–54	20	—
T. O.	20 mos.	Diarrhea (3–16–54)	typhimurium	3–26–54	160	10
				4– 2–54	160	—
				4– 4–54	160	—
P. M.	11 mos.	Diarrhea (7–12–54)	Derby	7–17–54	40	—
				7–20–54	10	—
B. R.	11 mos.	Diarrhea (5–21–54)	typhimurium	5–27–54	20	—
				6– 2–54	10	—
M. Z.	4 days	Diarrhea (7–19–54)	Oranienburg	7–22–54	20	—
				7–28–54	20	—
J. S.	2 yr.	Diarrhea (1–3–55)	typhimurium	1–13–55	160	—

* = *Reciprocal.*
** = *Less than 10.*
*** = *Not done.*

TABLE II

SALMONELLA HEMAGGLUTININS AND HEMOLYSINS IN SERUM OF PATIENT WITH *Salmonella schottmuelleri* INFECTION

Blood cells	Hemagglutinin Titers*	Hemolysin Titers*
Modified with *Salmonella*:		
Human O	1600	—**
Sheep	1600	100
Not modified:		
Human O	—	—
Sheep	—	25

* *Reciprocal.*
** *Less than 25.*

modification of the above *Salmonella* hemagglutination test is less sensitive than the hemagglutination method. Whether the use of complement from another source may enhance the sensitivity of the hemolysis method remains to be elucidated.

It was deemed to be of interest to determine whether patients with *Salmonella* infection respond also with an increase in heterologous bacterial hemagglutinins. To this end, the titers of hemagglutinins against various groups or types of *Salmonella*, *Shigella*, and *Escherichia* were determined in serum specimens from various patients. The results are recorded in Table III.

It may be noted that the patient responded with an increase in the titer of hemagglutinins against all organisms, but that the homologous antibody titers were far higher than those of heterologous antibodies. This observation, made in other instances as well, raises the question as to the specificity of the antibody response in patients with salmonellosis. Accordingly, absorption studies were carried out. Table IV shows the results of a typical experiment.

Table IV reveals that red blood cells modified with either *Salmonella tennessee* or *Shigella flexneri* absorb only homologous antibodies and thus produce a specific reduction in the titer. These findings suggest that certain patients with *Salmonella* infections respond with the formation of homologous antibodies (hemagglutinins) in high titer and of heterologous antibodies in considerably lower titer.

The Polyvalent Enterobacterial Hemagglutination Test

It was previously shown (10) that two crude antigens obtained from different serogroups of *E. coli* can be simultaneously or consecutively adsorbed on red blood cells. Evidence was presented to the effect that each red cell adsorbs both antigens and thus becomes reactive with either or both antibodies. It was deemed of interest to determine whether purified *E. coli* lipopolysaccharides behave in like fashion. This was shown to be the case. Table V gives the results of a typical experiment in which three different antigens, both crude and purified, were used.

TABLE III

HOMOLOGOUS AND HETEROLOGOUS HEMAGGLUTININ RESPONSE OF A PATIENT WITH *S. choleraesuis* INFECTION

	Titer* of hemagglutinins against						
	Salmonella			*Shigella*		*Escherichia coli*	
Date of serum	*choleraesuis* (VI, VII)	*typhi-murium* (IV, XII)	*muenchen-oregon* (VI, VIII)	*sonnei*	*flexneri*	O111	O55
10- 4-54	50	—**	—	—	—	—	—
10- 5-54	50	—	—	—	—	—	—
10- 8-54	1600	100	200	50	50	—	50
10-19-54	1600	50	100	50	50	25	25

* *Reciprocal.*
** *Less than 25.*

TABLE IV

HEMAGGLUTININ ABSORPTION TEST

Serum	Titer* of hemagglutinins against	
	Salmonella tennessee	*Shigella flexneri*
Unabsorbed	400	100
Absorbed with:		
S. tennessee	25	100
S. flexneri	400	25

* *Reciprocal.*

Perusal of this table shows that all three lipopolysaccharides, like the crude antigens, were simultaneously adsorbed on red blood cells. It may be pointed out that the minor cross-reactions obtained with the crude antigens and *E. coli* 111 serum were not encountered with the purified lipopolysaccharides.

On the basis of these and previous results it was deemed of interest to determine whether a polyvalent enterobacterial hemagglutination test might be useful in the screening of sera from patients for the detection of antibodies. Preliminary to such an investigation, experiments were undertaken in which crude *Salmonella* and *Shigella* antigens were used, singly and in combination, for modification of red blood cells. Promising results were obtained. Table VI presents the data from a typical experiment.

Perusal of this table clearly shows that the titers of homologous *Salmonella* and *Shigella* antibodies in the serum specimens from four patients with salmonellosis or shigellosis were identical in the monovalent and polyvalent hemagglutination tests. Thus, a mixture of crude antigens used in conjunction with the hemagglutination technique proved to be entirely adequate for the detection and titration of the corresponding antibodies. Further studies on this polyvalent hemagglutination test with purified antigens obtained from the major groups of *Salmonella*, *Shigella*, and enteropathogenic *Escherichia* are now in progress.

Discussion

The present investigation revealed that the hemagglutination technique is a sensitive method for the detection of *Salmonella* antibodies and distinctly superior to the conventional bacterial agglutination test. Of the 11 patients with bacteriologically proved salmonellosis, eight developed or had a high titer of homologous hemagglutinins provided that two serum specimens were available for study, one obtained within the first week of illness and the other at a later time. It is interesting to note that the remaining three patients were infants under one year of age. The question of immunological immaturity thus arises and deserves further study. The demonstration of a *Salmonella*

TABLE V

MONOVALENT AND POLYVALENT *Escherichia coli* HEMOLYSIS TEST

E. coli serogroup	OB antisera dilution (reciprocal)	Hemolysis by *E. coli* antisera of sheep erythrocytes modified with: Heated *E. coli* lipopolysaccharides 111 5μgm.	55 6.6 μgm.	26 20 μgm.	Mixture	Heated *E. coli* culture supernates 111 1/100	55 1/100	26 1/75	Mixture
111	200	4	–	–	4	4	2	1	4
	400	4	–	–	4	4	1	–	4
	800	4	–	–	4	4	–	–	4
	1600	4	–	–	4	3	–	–	3
	3200	4	–	–	4	1	–	–	1
	6400	2	–	–	2	–	–	–	–
	12,800	–	–	–	–	–	–	–	–
55	200	–	4	–	4	–	4	–	4
	400	–	4	–	4	–	4	–	4
	800	–	4	–	4	–	4	–	4
	1600	–	4	–	4	–	4	–	4
	3200	–	3	–	2	–	3	–	3
	6400	–	1	–	1	–	–	–	–
	12,800	–	–	–	–	–	–	–	–
26	100	–	–	4	4	–	–	4	4
	200	–	–	4	4	–	–	4	4
	400	–	–	3	4	–	–	4	4
	800	–	–	2	3	–	–	4	4
	1600	–	–	±	±	–	–	4	4
	3200	–	–	–	–	–	–	4	4
	6400	–	–	–	–	–	–	1	3
	12,800	–	–	–	–	–	–	–	–
	0	–	–	–	–	–	–	–	–

TABLE VI

MONOVALENT AND POLYVALENT ENTEROBACTERIAL HEMAGGLUTINATION TESTS

Patient	Infection	Titer* of hemagglutinins against				
		Salmonella muenchen-oregon	*Salmonella choleraesuis*	*Salmonella schottmuelleri*	*Shigella sonnei*	Mixture of all antigens
Dr. M.	*Salmonella muenchen-oregon*	*6400*	400	400	<200	*6400*
J. T.	*Salmonella choleraesuis*	400	*12,800*	200	<200	*12,800*
P. F.	*Salmonella Schottmuelleri*	200	200	*1600*	<200	*1600*
P. W.	*Shigella sonnei*	400	200	400	*3200*	*3200*

* *Reciprocal.*

hemagglutinin response offers a number of clinical applications. In the first place, it may be possible to differentiate between true salmonellosis and an infection, such as diarrheal disease, caused by an unrelated agent, for example a virus, in a *Salmonella* carrier. Secondly, the method may eventually make possible epidemiological studies of salmonellosis with particular reference to mild and subclinical infections in a household or community. In fact, in several instances we have been able to demonstrate high titers of hemagglutinins in the serum of siblings or parents of some of our patients, suggesting that they too had been infected.

It is now established that a variety of antigens from enteric pathogens can be adsorbed on red blood cells, namely, *Salmonella*, including *S. typhosa* (1, 2, 4, 7, 8, 21, 22, 23, 24, 25), *Shigella dysenteriae* (Shiga) (11), *Shigella flexneri* (3), *Shigella sonnei* (13), enteropathogenic *Escherichia coli* (9, 10, 12, 20, 21), and *Vibrio comma* (5). Thus it appears likely that the hemagglutination method may eventually be applied to serological diagnosis of all major bacterial enteric infections.

Since relatively large numbers of groups comprise the genera *Salmonella*, *Shigella*, and enteropathogenic *Escherichia coli*, much labor may be saved if the polyvalent hemagglutination test, described in this communication, is applied to the screening of serum specimens. Instead of using several antigens separately, as is necessary in the conventional bacterial agglutination test, it is possible to prepare a polyvalent antigen by modifying red blood cells with eight and possibly more enterobacterial antigens. Standardization of such a method may be possible through the use of purified enterobacterial lipopolysaccharides. Such studies are now under way in our laboratories.

A brief comment may be made regarding the observation of a rise, minor in degree, of heterologous antibodies during or following salmonellosis. It was shown that the homologous and heterologous antibodies could be clearly differentiated in absorption experiments. It will be interesting to determine whether increases in heterologous antibodies usually occur in various enteric infections. In this connection it is interesting to point out that it was shown in a recent study (15) that the percentage of random individuals, whose serum contains hemagglutinins against enteropathogenic *E. coli*, increases with age. The titers of these antibodies, however, are relatively low. The question presents itself as to whether the presence of such antibodies in relatively low titer is the result of infection with the homologous organisms, exposure to the identical antigens present in some unrelated material, or the result of stimulation by heterologous antigens.

The hemagglutination method may also be useful in the elucidation of the nature of various enterobacterial antigens. For example, Table III shows that a patient with *S. choleraesuis* infection developed antibodies against the homologous organism, containing VI and VII antigens, in high titer (1 : 1600) and heterologous antibodies against *S. muenchen-oregon*, containing VI and VIII antigens, in low titer only (1 : 200). This finding indicates that the

major antibody component is directed against antigen VII. It will be interesting to determine whether such antigens represent different molecules or a single molecule with different reactive groupings. A similar problem exists with respect to the O and B antigens of enteropathogenic *E. coli*.

Finally, it may be appropriate to point out that for the examination of human sera red blood cells of man (blood group O) are the material of choice. If sheep cells were to be used in the hemagglutination test, the patient's serum must be absorbed first with sheep red blood cells, in order to remove normal sheep agglutinins. Furthermore, the hemolytic modification of the enterobacterial hemagglutination test is less sensitive than the hemagglutination technique, when guinea pig complement is used. This is in sharp contrast to the findings with antisera prepared in rabbits. The reason why human red blood cells modified with enterobacterial antigen are readily agglutinated by homologous bacterial human antibodies but not at all lysed in the presence of guinea pig complement remains to be elucidated.

Acknowledgment

The authors are indebted to Dr. Ivan Saphra of the National Salmonella Center for type determinations of the *Salmonella* strains.

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THE EFFECTS OF THE IONIC ENVIRONMENT ON THE CHROMATIN STRUCTURES OF BACTERIA[1]

By J. F. Whitfield[2] and R. G. E. Murray

Abstract

The aggregation of bacterial chromatin into compact masses under a variety of circumstances (exposure to high salt concentrations, low temperature, ultraviolet irradiation, metabolic inhibitors, and starvation) is a function of the concentration of electrolytes in the environment of the cell. This effect can be prevented or reversed in an environment deficient in salts. The observations indicate that chromatin aggregation is a consequence of interaction of cations (Na^+ and K^+, in particular) and polymerized desoxyribose nucleic acid, which behaves as an anionic gel. The organisms used (*Shigella dysenteriae*, *Escherichia coli*, *Bacillus cereus*, *Caryophanon latum*, a wild yeast, and a *Geotrichum* sp.) were able to maintain their "normal" configuration of chromatin in salt-deficient media and in media with up to 3% salt as long as their metabolism was intact. Disruption of metabolism by exposure to cold or metabolic inhibitors produced aggregation or dispersion of chromatin according to the ionic environment. This range of change in nuclear form can be produced in the living cell without apparent or permanent damage to viability. These studies indicate that bacterial and fungal cells share with cells of higher organisms the ability to regulate the influx and efflux of cations. Chromatin serves as a sensitive indicator of the integrity of these ion regulatory mechanisms. Some implications of these observations are discussed.

Introduction

It is a common observation that the arrangement of chromatin in a bacterial cell can vary from compact aggregates to very diffuse granular inclusions. This variability of internal structures has been largely avoided in recent years by the study of individual species, as test objects, under defined conditions of growth and nutrition; this has led to fair general agreement on what might be considered the "normal" configuration of chromatin within these restricted conditions. There is no apparent understanding of the "abnormal" configurations and the reasons for their appearance. The most common of these abnormalities is the condensation of chromatin structures into compact rounded aggregates, which may fuse along the axis of the cell to form an axial filament. This phenomenon has been observed as a response to imposed conditions such as the exposure of bacteria of various genera to certain bacteriophages (3, 20, 21, 39) to antibiotics (9, 20), to metabolic inhibitors such as 2,4-dinitrophenol (18), and to culture aging (4, 11).

It is probable that there is a common basis for the phenomenon of chromatin aggregation and fusion, despite the diversity of precipitating conditions.

[1]*Manuscript received July 29, 1955.*
Contribution from the Department of Bacteriology and Immunology, University of Western Ontario, London, Ontario. The work was made possible by grants from the National Research Council of Canada. This work formed part of a thesis (39) in which some of the experiments are considered in greater detail.
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The only common factor is a cessation of growth, but this does not provide an explanation of the phenomenon. A basis for controlled experiment is provided by the observation of Johnson and Gray (19) that high concentrations of sodium chloride caused nuclear aggregation and axial filament formation in *Achromobacter fischeri*. Most importantly, they showed that this effect was reversible and thus indicated that salts might be an important environmental factor contributing to variation in chromatin arrangement.

This paper presents experiments to define the factors involved in the phenomenon of nuclear aggregation and fusion. A mechanism is proposed that appears to correlate many of the various observations reported in the literature.

Materials and Methods

Two strains of *Shigella dysenteriae*, Sh and Sh/s, were used in the majority of experiments. These strains originally served as indicator strains in our cytological studies of the process of lysogenization (40), in which chromatin aggregation was a prominent feature. Many experiments were repeated or extended using *Bacillus cereus*, *Escherichia coli* B, *E. coli* K12, *Geotrichum* sp., a wild yeast, and *Caryophanon latum*.

Representative experiments are reported in this paper. Unless otherwise indicated, the medium used for all studies was a tryptone – yeast extract medium, which is the LB medium used by Bertani (2) with the omission of the sodium and calcium chlorides. This was a convenient basal medium because flame photometry showed that it contained relatively low concentrations of sodium (20.5 meq./liter Na^+) and potassium (11 meq./liter K^+) ions; various salts were added as required. The medium was made up with agar (Noble agar, Difco, 1%) for most experiments.

The transfer of cells to a new environment was most conveniently and accurately carried out by making impression transfers from an agar block onto a medium of modified composition. Details will be given in the experimental section.

For observation, the cells were fixed *in situ* on blocks of the agar medium for 90 sec. in the vapor over 2.0% osmium tetroxide before impression preparations were made on cover slips. These were hydrolyzed in *N* hydrochloric acid at 60°C., and stained with Giemsa and with SO_2-azure A according to the methods previously described (25, 26). The cells in many experiments were checked by phase-contrast microscopy; so that it may be reasonably assumed that the observations are not vitiated by gross artifacts of preparation.

Preparations were examined with a 2 mm. apochromatic (Leitz) objective lens (N.A. 1.32), an aplanatic condenser (N.A. 1.4), a 15× eyepiece, and Köhler illumination. Panatomic X (Kodak) cut film and, in most cases, an interference filter (5390 Å, Baird Associates) were used in photography.

Experimental

The Effects of a High Salt Concentration on Chromatin Arrangement

The observations of Johnson and Gray (19) were confirmed using *S. dysenteriae* grown for three hours on LB medium at 37°C. Blocks bearing the organism were supported in a 1.7 *M* solution of sodium chloride so that the cell bearing surface was exposed; these were removed at intervals to follow the effects. The chromatin bodies gradually lost their normal configuration and became dense rounded aggregates which in turn fused into an axial filament of chromatin (Figs. 1, 2, and 3).

However, a drawback to this type of experiment is that materials from the LB agar leach into the surrounding salt solution along with entry of salt into the agar. This was avoided by transferring the cells alone to a new environment of the desired constitution: A block of normal agar bearing three-hour cells was inverted onto the high salt (1.7 *M*) agar and immediately flicked off leaving an "impression transfer". No leaching will take place under these circumstances. A comparison of Tables I and II shows that the rate of aggregation is considerably reduced with the block transfer method as opposed to the modified Johnson and Gray procedure.

TABLE I

NUCLEAR AGGREGATION IN CELLS OF *S. dysenteriae* WITH EXPOSURE TO 1.7 *M* NaCl

Time of exposure	% showing no aggregation to slight aggregation	% showing partial aggregation to complete aggregation	Total number of cells counted
30 min.	78.5	21.5	256
90 min.	48.8	51.2	219
120 min.	2.9	97.1	344

TABLE II

NUCLEAR AGGREGATION IN CELLS OF *S. dysenteriae* ON LB AGAR CONTAINING 1.7 *M* NaCl

Duration of exposure	% showing no aggregation to slight aggregation	% showing partial aggregation to complete aggregation	Total number of cells counted
30 min.	96.2	3.8	215
60 min.	92.0	8.0	225
90 min.	90.5	9.5	264
120 min.	91.6	8.4	228

When salt-treated cells (cells exposed for two hours to 1.7 *M* aqueous solution of sodium chloride) were placed into a solution of distilled water, the chromatin aggregates showed fragmentation. However, this procedure was difficult to control, because of further leaching of nutrients and salts, and the impression transfer method was used. The axial filaments segregated into granules and bars of chromatin (Fig. 4) and these slowly moved out to the periphery of the cell. The larger bars of chromatin fragmented into smaller discrete granules, showing progressive reorganization of the chromatin into normal nuclear structures (Fig. 5). The entire process required 70–90 min. for completion.

This reversal is obviously a consequence of simple removal of salts from the environment. The implication of cellular metabolism as a factor in this process was demonstrated and the following experiment is an example.

B. cereus was grown on heart infusion agar for two and one-half hours at 37°C. These cells were transferred, using the block transfer technique, to warmed plates of heart infusion agar with no added sodium chloride (i.e. the same medium) and to heart infusion agar containing sodium chloride at a concentration of 3%. On the agar containing no added salt, the cells were cytologically normal and showed uninterrupted growth. On the heart infusion agar with added salt, the nuclei showed condensed and aggregated chromatin during the first five minutes following transfer from the normal agar, and cell growth ceased. However, by 20 min. after transfer, the growth resumed and the chromatin aggregates showed signs of rearrangement. Between 30 and 60 min. the chromatin pattern was indistinguishable from the controls. This series is illustrated in Figs. 6, 7, and 8.

The foregoing observations indicate that a high salt concentration in the environment will cause nuclear condensation and aggregation. It is also evident that some factor or factors related to metabolism also intervene in this process. The metabolic factors will be discussed later on.

Either the cation or the anion or both might participate in the process. Sodium acetate (1.7 *M*) produced nuclear aggregation in *S. dysenteriae* at the same rate and with the same cytological characteristics as that with sodium chloride (1.7 *M*). The calcium salts (calcium chloride and calcium acetate, 0.9 *M*) caused the appearance of axial filaments but they were much shorter and thicker. In addition, the entire protoplast contracted and pulled away from the cell membrane, which was not observed with the salts of

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FIG. 1. Normal *S. dysenteriae* (Sh) grown for three hours on LB + 0.17 *M* sodium chloride at 37°C. This represents what is referred to as the "normal configuration" of chromatin for this organism in the early exponential phase. This, and all the other figures, is an HCl–Giemsa preparation. Azure-SO_2 preparations are similar but more delicate. The magnification in all figures approximates that shown.

Fig. 2. Sh following 30 min. exposure of agar block to 1.7 *M* sodium chloride solution.

FIG. 3. Sh following 125 min. exposure of agar block to 1.7 *M* sodium chloride solution.

FIG. 4. Sh, 45 min. recovery on normal LB agar at 37°C. Some cells still show fully aggregated chromatin.

FIG. 5. Sh, 90 min. recovery on normal LB agar at 37°C.

PLATE I

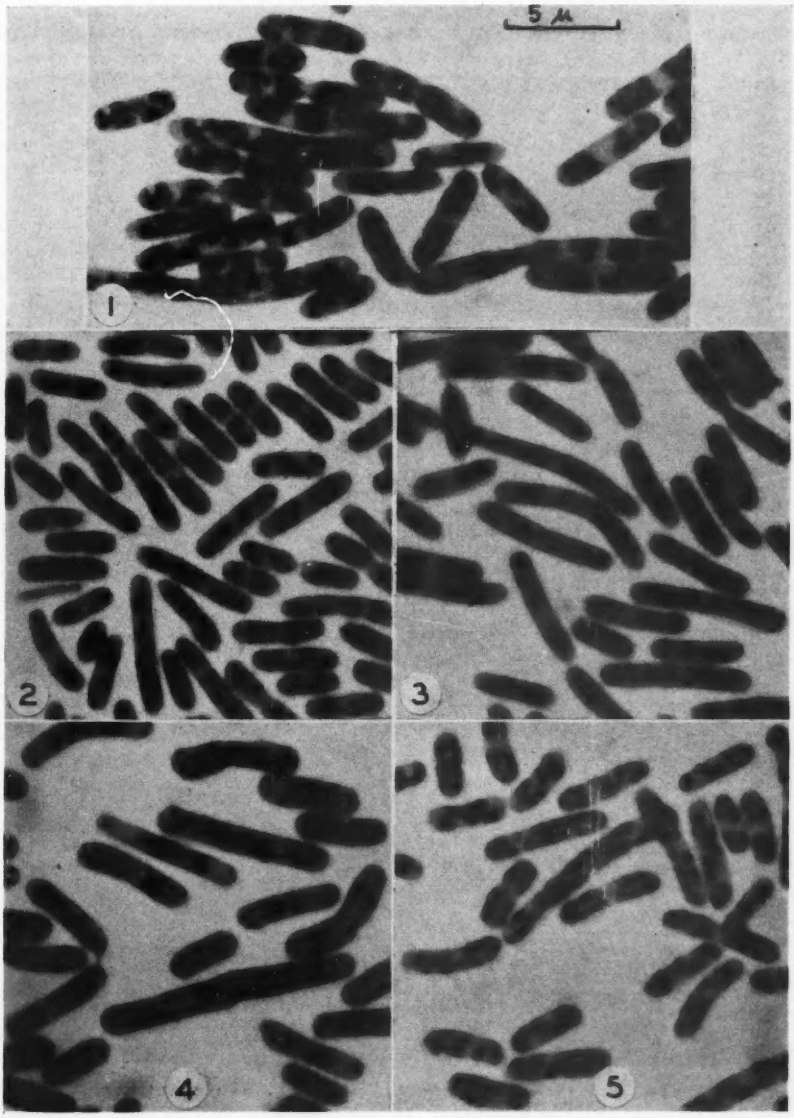
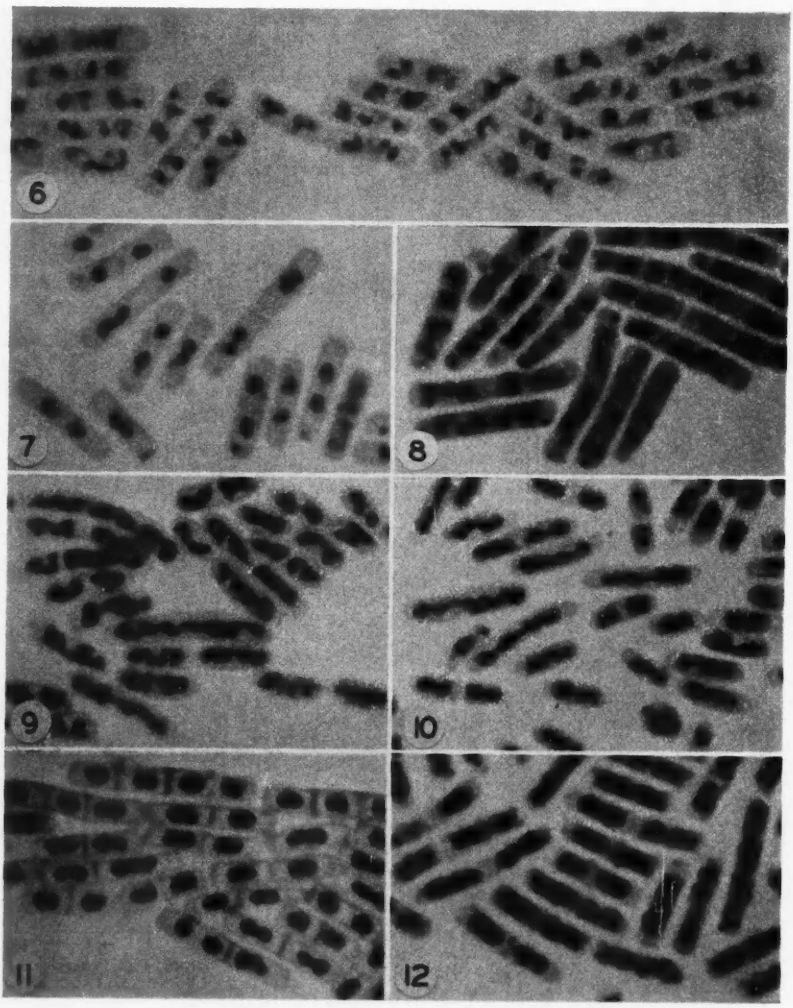


PLATE II



monovalent cations. The cytological pictures of aggregation due to potassium chloride, potassium acetate, and lithium chloride were essentially the same as that of aggregation due to sodium chloride. Thus, it appears that the cation is the determinant, since exchanging the anion does not alter the result.

It has been stated above that metabolism plays an important role in nuclear aggregation. In the following experiments the nature of this role will be defined.

The Effects of Cold

Flynn and Maizels (16) and other workers in mammalian physiology have found that the ionic balance of cells is disturbed by lowering the temperature of the environment. The selective accumulation of potassium and excretion of sodium are inhibited as a consequence of metabolic inhibition by low temperature. If axial filament formation is a consequence of a derangement of electrolyte metabolism, then exposure to cold might result in filament formation and the cells should regain their normal nuclear morphology at some time following their return to 37°C.

S. dysenteriae was grown for three hours at 37°C. on LB + 0.17 *M* sodium chloride agar and then transferred to the same medium which had been cooled to 4°C. After one hour at low temperature, the nuclear structures showed aggregation. By two hours, spherical aggregates and axial filaments had appeared (Fig. 9). By three to four hours, the nuclear structures had become completely aggregated throughout the entire population (Fig. 10), and a small proportion of the cells underwent lysis. By 20 hr., the population was uniform and consisted of cells with clearly defined axial filaments.

Similar experiments were performed on cells of the fungus *Geotrichum*, a wild yeast strain, and *Caryophanon latum*. In every case, the chromatin underwent aggregation on exposure to cold on LB + 0.17 *M* sodium chloride agar.

B. cereus behaved differently. It was grown on LB + 0.17 *M* sodium chloride for two and one-half hours at 37°C. and transferred to the cold as described above. During the first 30–60 min. at 4°C., the nuclei of the cells aggregated into short axial filaments (Fig. 11). This was only a transient effect and was followed during the next three hours by expansion and fragmentation of the axial filaments (Fig. 12). This fragmentation appeared

FIG. 6. *B. cereus*, two and one-half hours on heart infusion agar at 37°C. Control preparation to Figs. 7 and 8.

FIG. 7. Companion to Fig. 6, five minutes after transfer to medium containing 3% sodium chloride.

FIG. 8. Companion to Fig. 6, 30 min. after transfer to medium containing 3% sodium chloride.

FIG. 9. Sh one hour after transfer to 4°C. on LB + 0.17 *M* sodium chloride medium.

FIG. 10. Sh, as Fig. 9, four hours after transfer.

FIG. 11. *B. cereus* after one hour at 4°C. The starting material showed similar configuration to Fig. 6.

FIG. 12. *B. cereus*, as Fig. 11, four hours after transfer.

TABLE III

THE EFFECT OF RAISING THE SODIUM CHLORIDE CONCENTRATION ON NUCLEAR AGGREGATION IN Sh/s AT 4°C.

Medium	% of cells showing*		Total number of cells counted
	Complete nuclear aggregation	Partial nuclear aggregation	
LB + 0.17 *M* NaCl	17.4	82.6	304
LB + 0.34 *M* NaCl	83.3	16.7	209

**These results were recorded following one hour exposure to cold.*

to be lethal to the cells since it was followed, in most instances, by lysis. Thus, *B. cereus* does not allow its nuclei to remain in the aggregated state for very long, while in *S. dysenteriae* and the other cells studied, the axial filaments remain as long as they are in the cold environment in the presence of salt.

Since the ionic components of the cells should tend to equilibrate with those of the medium when metabolism is halted or slowed down, then raising the ionic concentration of the medium should result in a more rapid appearance of axial filaments and, conversely, lowering the ionic concentration of the medium below normal should delay filament formation. Cells of *S. dysenteriae* grown on LB agar + 0.17 *M* sodium chloride for three hours at 37°C. were transferred to duplicate plates of three LB media: LB + 0.0, 0.17, and 0.34 *M* sodium chloride. One of each pair was incubated at 37°C. and the other refrigerated at 4°C.

Cells growing on each of the three LB media at 37°C. were cytologically normal. At 4°C. there was nuclear aggregation on salted media and the rate of aggregation increased with the salt concentration. In Table III, the proportion of cells affected in a typical experiment are recorded.

The cells refrigerated for 3–20 hr. on the LB medium without added sodium chloride, which had the normal potassium and sodium levels (11.4 and 20 meq./liter), showed no nuclear aggregation. Instead, the chromatin underwent fragmentation (Fig. 13). These observations were extended to include cells of *Caryophanon latum* and a wild yeast. In these cells, chromatin aggregation at 4°C. was prevented on salt-deficient media and was replaced by expansion and fragmentation of the chromatin structures.

FIG. 13. Sh after three hours at 4°C. on LB medium without added salt.

FIG. 14. Sh after 20 hr. at 4°C. on LB + 0.17 *M* sodium chloride.

FIG. 15. Sh, as Fig. 14, 15 min. after transfer to an identical medium at 37°C.

FIG. 16. Sh, as Figs. 14 and 15, 45 min. after transfer to 37°C.

FIG. 17. Sh, transferred from salted medium (20 hr. at 4°C.) to salt-deficient medium at 4°C.

PLATE III

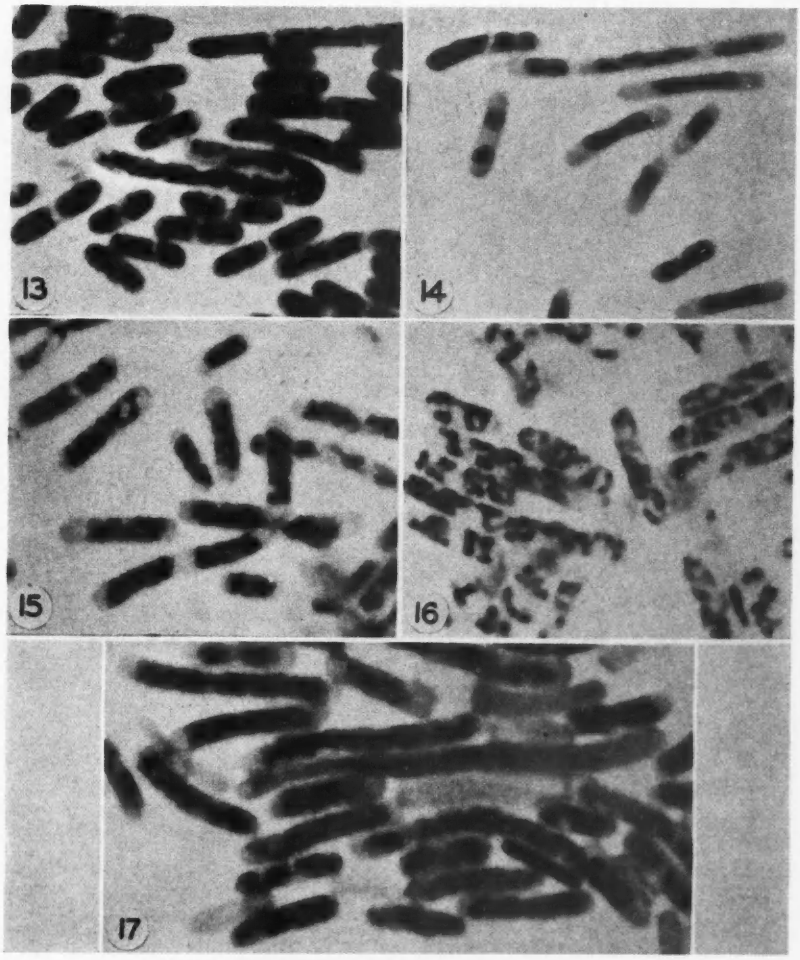
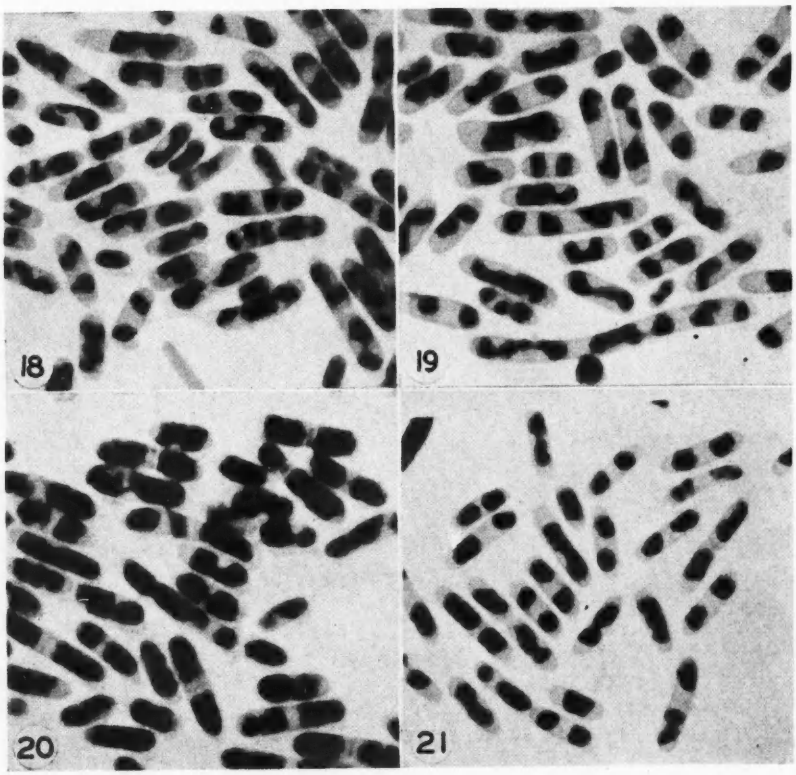


PLATE IV



Cells of *S. dysenteriae* showing axial chromatin filaments (LB + 0.17 *M* sodium chloride, 4°C. for 20 hr., Fig. 14) were removed from the refrigerator and transferred to the surface of an identical medium warmed to 37°C. The axial filaments showed expansion within 5–10 min. The chromatin crust of the filaments showed fragmentation into discrete bars and granules which were later found at the periphery of the cell (Fig. 15). The expanding reticulum became segregated into normal or nearly normal nuclei, and by 45 min. (Fig. 16) the process was complete and the cells began to divide. It should be noted that the cells resumed the normal (i.e. early exponential growth phase) cytological appearance although they had been cooled for 20 hr.

In order to determine whether cold aggregation is reversible *in the cold* by lowering the salt concentration in the medium, cells of Sh/s were plated on LB + 0.17 *M* potassium chloride and LB + 0.17 *M* sodium chloride agars. The cells were cooled on these media to 4°C. and held at that temperature for 20 hr. In these populations the nuclei became aggregated (Fig. 14). Both populations were then transferred, in the cold, to LB with no added salts and only the basal sodium and potassium concentrations. On this deficient agar, both groups showed an extensive fragmentation of their axial filaments and movement of the granules to the periphery of the cell. The fragmentation and peripheral movements were accompanied by swelling and by lysis in a large proportion of the cells (Fig. 17).

It was important to determine whether the fragmented nuclear structures in cells held on salt-deficient agar at 4°C. were capable of returning to normal. A plate of cells in this state, after 14 hr. refrigeration was warmed to 37°C. and the nuclear changes were followed.

At the start the nuclear structures of the cell were fragmented and appeared as masses of fine chromatin granules and remained so for 10 min. after transfer to 37°C. By 20 min., reticula of granules and threads appeared in the granular masses. After 30–45 min. the reticula were more clearly defined and the fine granules had completely disappeared, apparently having been incorporated into the normal reticulum. The cells appeared to have regained their normal chromatin configurations within an hour. It must be emphasized that this recovery took place on salt-deficient agar.

Successful recovery was not the fate of every individual in the population. Numerous cell 'ghosts' were present in the 30 min. preparations. However, the greater part of the population did recover to normal growth and structure.

Cells exposed to 4°C. for 14 hr. on salt-deficient agar were transferred to LB agar + 0.17 *M* sodium chloride at 37°C. The first change, 10 min. after transfer, was the aggregation of the chromatin granules into axial filaments.

FIG. 18. Sh after two minutes' ultraviolet irradiation on LB + 0.17 *M* sodium chloride medium.

FIG. 19. Sh, as Fig. 18, after five minutes' irradiation.

FIG. 20. Sh after eight minutes' ultraviolet irradiation on LB medium without added salt.

FIG. 21. Sh, three minutes after transfer in dark to LB + 0.17 *M* sodium chloride medium after six minutes' ultraviolet irradiation on LB medium without added salt.

TABLE IV

THE EFFECTS OF VARIOUS CONCENTRATIONS OF POTASSIUM CHLORIDE IN THE MEDIUM ON CHROMATIN AGGREGATION IN *S. dysenteriae* IN THE COLD

% K^+ *added* to LB agar	Changes in nuclear structure at 4°C. after:	
	4 hr.	24 hr.
1.3 (170)*	100% Aggregation	Partial and complete aggregation
0.9 (115.6)	Aggregation + partial aggregation	Partial aggregation
0.8 (104)	Aggregation + partial aggregation	Fragmentation
0.6 (78)	Partial aggregation + some fragmentation	Fragmentation
0.5 (65)	Some aggregation although mainly incipient fragmentation	Fragmentation
0.4 (52)	Some aggregation and incipient fragmentation	Fragmentation
0.2 (26)	Incipient fragmentation	Fragmentation
0.1 (13)	Fragmentation	Fragmentation

**The figures in brackets signify meq./l. of K^+ added. The basal medium contains 11 meq./l. K^+ and 20.5 meq./l. of Na^+. Hence, the total K^+ concentration is obtained by adding 11 to the figures in the brackets.*

In the major part of the population aggregation was not complete. By 20 min. rodlets of chromatin and triangular configurations of granules made their appearance. By 45 min., the normal chromatin pattern had been established. Some lysis took place during the first 10 min. after transfer.

Salt Titration

The sensitivity of bacterial chromatin to changes in the ionic environment suggested a means of titrating the internal free cation concentration using the chromatin of the cell as an indicator. Metabolic complications were largely avoided by running the experiments at 4°C. Cells of *S. dysenteriae*, grown for three hours on LB medium were transferred to the chilled media containing various concentrations of salts. Preparations were taken at intervals thereafter to follow the effects on chromatin configuration, which was perfectly normal at the outset.

The first experiments concerned the effects of various extracellular potassium concentrations on the nuclear structures at 4°C. The results of such an experiment are recorded in Table IV and show that (1) a potassium concentration of over 181 meq./liter is required for formation and maintenance of nuclear aggregates, (2) that a concentration of potassium around 126 meq./liter is required to prevent fragmentation, (3) a concentration of potassium less than 89 meq./liter is required for immediate fragmentation as opposed to partial aggregation followed by fragmentation.

In a similar fashion, LB agar plates having various concentrations of sodium chloride were prepared and normal three hour cells were transferred to these plates and cooled to 4°C. for 18 hr. From the results recorded in Table V, it may be seen that the concentration of sodium chloride in the medium has to be considerably reduced before aggregation is prevented.

TABLE V

THE EFFECTS OF VARIOUS CONCENTRATIONS OF SODIUM CHLORIDE IN THE MEDIUM ON NUCLEAR AGGREGATION IN Sh/s IN THE COLD

% sodium chloride *added* to LB agar	Changes in nuclear structure by 18 hr. at 4°C.
0.70 (119)*	Aggregation
0.60 (102)	Aggregation
0.50 (85)	Aggregation
0.40 (68)	Aggregation
0.30 (51)	Aggregation (partial)
0.20 (34)	Aggregation (partial)
0.10 (17)	Aggregation and fragmentation. Normal nuclei seen in some cells
0.05 (8.5)	Fragmentation with many cells retaining their normal nuclear structures

**Figures in brackets signify meq./l. of Na^+ added. The basal medium contained 20.5 meq./l. of Na^+ and 11 meq./l. of K^+.*

Culture Aging

Delaporte (11), Bisset (4), and others have noted chromatin aggregation and axial filament formation in older cultures of many bacteria. In this situation, active growth and metabolism have ceased or are much reduced. It would be expected that active osmoregulation would also diminish in effectiveness and sufficient free cations would accumulate within the cell for nuclear aggregation to take place.

In older broth cultures (16–21 hr.) of both *S. dysenteriae* and *E. coli*, the nuclei appear as short, tightly compacted axial filaments or annuli. This situation applies to cells incubated in aerated LB + 0.17 *M* sodium chloride broth. In aerated LB broth with no added salt, the nuclei in cells from older cultures were observed to have undergone fragmentation and appear as diffuse masses of chromatin. When these cells were transferred to fresh salt-deficient broth *or* agar, the fragmented chromatin structures became reorganized into the compact nuclear structures characteristic of cells from young cultures in normal media. This acquisition of normal structure was accompanied by the commencement of growth and, presumably, by the mobilization of the metabolic means to capture and retain necessary ions.

In older cultures, growth will cease because of many factors among which are exhaustion of available nutrients and adverse shifts in pH.

Starvation

Kellenberger (20) noted that transfer of cells of *E. coli* K12 from young cultures in nutrient synthetic medium to the same medium lacking the carbon source (glucose or lactate), resulted in nuclear aggregation. We have confirmed this observation.

The Effects of pH

B. cereus was grown on tryptose agar at pH 7.0 for two and one-half to two and three-quarter hours at 37°C. Transfer of these cells to tryptose agar at pH 8.0, 9.0, and 10.0 was carried out using the block transfer method. The cells were held at each pH value for 60 min. at 37°C. and then fixed, hydrolyzed, and stained. At pH 8.0 a few axial filaments were observed. At pH 9.0 the proportion of cells with axial filaments was higher but these did not comprise the majority of forms in the whole population. The axial filament became the predominant form at pH 10.0. Cells that had been on tryptose agar at pH 10.0 for 60 min. were transferred back to tryptose agar at pH 7.0. By 15 min. after transfer, all or virtually all of the axial filament types had recovered the normal or near-normal nuclear patterns.

Young cells of *S. dysenteriae*, grown on LB + 0.17 *M* sodium chloride at pH 7.2, were transferred at 37°C. to media of identical constitution but having pH values ranging from 5.0–10.0. At low pH values no change in chromatin configuration resulted. At pH 9.0, the cells grew in long chains and the nuclear structures still retained their reticular condition to some extent, although slight aggregation took place. At pH 10.0, the nuclei became aggregated during the first three hours into long thin axial filaments and rather small spherical aggregates. But after one hour a number of these axial filaments expanded and their walls became fragmented into discrete bars and granules, which accounts for the drop in percentage shown in the table. This stage was followed by destruction of the cell by lysis. The results are summarized in Table VI.

TABLE VI

THE EFFECT OF LB AGAR AT pH 10 ON THE NUCLEAR STRUCTURES OF *S. dysenteriae*

Duration of exposure	% of cells showing		Total number of cells counted
	Non-aggregated chromatin	Aggregated chromatin	
30 min.	84.6	15.4	332
60 min.	42.4	57.6	257
120 min.	85.0	15.0	248
150 min.	74.2	25.8	295

Metabolic Inhibitors

A trial of some metabolic inhibitors was important as a complement to the observations on the effect of cold. Furthermore, both 2,4-dinitrophenol (DNP) and some of the antibiotics (18, 9) have been shown to cause chromatin aggregation.

Only a limited experiment was performed with DNP (2.3×10^{-3} *M*) incorporated in Kellenberger's "nutrient F" medium (20). Young cells of *E. coli* K12 were suspended in the medium and aerated. In normal F medium, without DNP, no nuclear aggregation took place. In F + DNP, the nuclei aggregated into spherical aggregates or annuli and short axial filaments at about 15 min. after transfer. This experiment provided the clue that metabolism, expecially ATP, was essential for the maintenance of normal nuclear structure and that disturbance of this metabolic aspect alters this structure.

Antibiotics act as metabolic inhibitors. Kellenberger (20) and DeLamater (9) studied the cytological effects of oxytetracycline, chlortetracycline, streptomycin, chloramphenicol, and erythromycin. Although these antibiotics differ considerably in the chemical structure, they all caused nuclear aggregation.

S. dysenteriae was exposed to 500 μgm./ml. diyhdrostreptomycin incorporated into normal and salt-deficient LB agar. Nuclear aggregation appeared in every cell on the salt-containing agar. A large proportion of cells on salt-deficient agar did not form typical chromatin annuli but underwent nuclear fragmentation and lysis. A streptomycin resistant mutant (Sh/s) did not show any cytological changes on either the salt-sufficient or salt-deficient media containing streptomycin.

Ultraviolet Irradiation

When Kellenberger (20) studied the effects of ultraviolet irradiation on the nuclear structures of normal and lysogenic *E. coli* K12, he observed chromatin fragmentation. However, cytological studies on induction of lysogenic *B. megaterium* (12) indicated that axial filament production might result from irradiation and the more recent studies of Mudd and co-workers (24) on *E. coli* K12 showed that the axial filament preceded fragmentation under their conditions. Our experiments show that the conditions of the experiment will determine the cytological effect.

Irradiation was performed in a room incubator at 37°C. using a 15-w. General Electric germicidal lamp at a distance of 14 in. from the plate. Blocks were cut from the agar plates at intervals, while they were under the light, and immediately fixed.

Irradiation of *S. dysenteriae* (three-hour cells) on LB + 0.17 *M* sodium chloride resulted in the aggregation of nuclear structures. Irradiation for 30 sec. was sufficient to induce aggregation, giving the appearance of a peripheral fusion of granules and a thickening of the connecting chromatin threads. After one to two minutes' irradiation, the chromatin threads and granules had begun to collect into spherical aggregates (annuli) and after four minutes the process of aggregation was almost complete (Figs. 18, 19).

When cells were irradiated on LB with no added sodium chloride, the same sequence was not observed. The chromatin, by four minutes' exposure to the irradiation, although present in ragged, peripheral patches, showed little or no evidence of aggregation. As the duration of irradiation increased, the chromatin became definitely fragmented and lay in irregular masses of which

there were, on the average, two per cell (Fig. 20). The chromatin thus gave the impression of a mass of fine granules lying within the bounds of definitely delineated sites within the cell.

The effect of altering the ionic environment of the cell on the irradiation changes was clearly shown by transferring cells irradiated for six minutes on medium without added salt to LB + 0.17 *M* sodium chloride agar in the dark. On the salt-deficient agar, the irradiation caused nuclear fragmentation. However, the chromatin fragments collected into typical spherical aggregates and short axial filaments during the first four minutes following transfer to the normal LB agar (Fig. 21).

When cells, irradiated for various times on LB + 0.17 *M* sodium chloride, and showing chromatin aggregation, were removed from the light source into the dark, it was noticed that the nuclear aggregates fragmented. The degree of fragmentation following irradiation was a function of the duration of irradiation. The nuclear structures fragmented into fewer and larger granules following shorter duration irradiation than irradiation of greater duration. Fragmentation was followed by the appearance of cells without demonstrable chromatin and cells in which the chromatin granules had collected in the middle of the cell.

Discussion

It is evident that the degree of aggregation or dispersion of the chromatin of bacterial nuclei is determined, to a large extent, by the ionic environment and the metabolic capability of the cell. Experiments using different salts of sodium and calcium indicate that the cation and not the anion is responsible for this effect.

Chromatin (i.e. the specifically stainable part) is composed of desoxyribose nucleic acid (DNA) in a highly polymerized condition. At intermediate pH values, the charge of DNA should be negative since the isoelectric point lies around pH 2.0 (17). Butler (5) and Butler and James (6), studying the diffusion of polymerized DNA in aqueous solutions, concluded that DNA had a gel structure with a rigidity caused by the mutual repulsion of similarly charged particles. At low salt concentrations or in distilled water, the repulsion of the components results in swelling of the gel, while raising the concentration of cations lowers the repulsive forces and the gel shrinks.

Watson and Crick (37, 38) proposed a structure for DNA. According to their concept, the molecule is composed of a polynucleotide duplex with a helical configuration; the purines and pyrimidines are clustered about the axis of the molecule while the phosphorus groups are arranged around the outside. This structure should make the molecule more accessible to cations. Pauling and Corey (27) have proposed another structure for DNA but the observed physical behavior of DNA favors the Watson and Crick model.

These considerations indicate that DNA could interact with cations and shrink considerably. However, it is somewhat difficult to correlate the physical properties *in vitro* with those *in vivo*. Mazia (23) provided an

important hypothesis on the structure of insect chromosomes, which was arrived at by a similar experimental approach and appears to be readily adaptable to the present observations. He visualized these chromosomes as being composed of a large number of units, interlinked electrostatically by divalent cations such as calcium and magnesium and repelling one another because of their like (negative) charge. The greater the density of cations surrounding the structure, the greater would be the degree of neutralization of the surface charge on the units.

It is conceivable that bacterial chromatin structures also are made up of a number of small negatively charged units, whose degree of packing into solid structures would be a function of the density of cations surrounding them. That DNA is a prerequisite for reversible ion-induced contraction of chromosomes may be inferred from the results of Ris and Mirsky (29) who found that DNA-deficient lampbrush chromosomes of the frog oöcyte did not respond to salts as did chromosomes containing larger amounts of this nucleic acid.

The effect of alterations in the ionic environment is much less marked under conditions which are optimal for the maintenance of the cell's metabolic state, e.g. exposure of *S. dysenteriae* to 1.7 *M* sodium chloride in complete agar at 37°C. More suggestive is the observation that *B. cereus* responds with only transient chromatin aggregation when confronted with a high salt concentration in complete medium. The reversion to normality of the chromatin configuration would seem to indicate that the cell is able to attain an equilibrium with the new ionic environment and proceed to grow normally. This sequence of events could be due to a sudden flooding of the cell interior with sodium followed by an increase in the activity of some sort of metabolic sodium "pump". The action of cold, dinitrophenol, streptomycin, and ultraviolet irradiation in causing chromatin aggregation in cells on normal salt-containing media while no such aggregation appears in cells on low-salt media would also indicate that metabolism is maintaining normal ion-regulation.

In many types of cells there appears to be a metabolically conditioned accumulation of potassium by the cell and extrusion of sodium from the cell (1, 8, 16, 22, 28, 34, 35, 36). The accumulation of potassium by bacteria has been demonstrated (7, 13, 14, 15, 30, 31). A metabolism-linked exclusion of sodium from a bacterial cell (*Micrococcus halodenitrificans*) has been demonstrated by Robinson *et al.* (33).

Cold and metabolic inhibitors such as 2,4-dinitrophenol would reduce the effectiveness of the hypothetical sodium pumping mechanism and thus sodium would be able to enter the cell. However, it appears from the effects of cold on cells on media containing various concentrations of potassium chloride that some release of potassium from binding sites within the cell may occur. This release, together with any sodium and other cations entering from the medium, might be expected to produce a sufficient cation density for aggregation to occur.

On *salt-deficient* media, exposure to cold, antibiotics, and ultraviolet irradiation does not result in chromatin aggregation. Instead, the nuclear structures fragment and these may be the same type of fragments proposed by Mazia (23) for insect chromosomes. One must assume that the total cationic concentration in cold-treated or irradiated cells on salt-deficient media would be lowered considerably by diffusion into the medium. As the cationic level within the cell is lowered, the negatively-charged chromatin becomes dispersed owing to mutual repulsion of its units and, on this hypothesis, the nucleus will appear to fragment.

The fragmented nuclear structure in cells exposed to cold is capable of recovering its normal structure upon warming to 37°C. We propose that the resumption of normal metabolism allows the accumulation of potassium to gain a sufficient intracellular cation level for the formation of the more closely packed normal structures.

By similar argument, starvation (such as the lack of a carbon source) results in nuclear aggregation because the cell cannot provide sufficient energy to maintain normal ionic regulation. It is interesting to note that nuclear aggregation does not appear in cells from older cultures grown in salt-deficient agar or broth. The nuclei under these conditions were very diffuse, approaching the fragmented form. This diffuse nuclear material remained capable of giving rise to perfectly normal nuclear structures as soon as growth resumed as a result of subculture onto fresh medium. But this is probably only one of a series of factors involved in the nuclear aggregation observed in cells from older, stationary cultures. It is evident that the development of an adverse pH in the medium can be another factor.

The observation of reversible nuclear fragmentation would appear to bear out the idea of the existence of subunits of the bacterial chromatin. It is, as yet, impossible to define clearly the nature of these units and their orientation within the chromatin bodies of the normal nucleus.

The titration of the equilibrium point at which the ionic concentration inside and outside the cell will balance, judged by the retention of "normal" chromatin configuration, may be one way of deciding upon an isotonic medium for bacteriological purposes. However, the end point of the titration, as can be seen in Tables IV and V, cannot be expected to be very exact even with an ideal population of cells and is probably complicated by release of ions from binding sites in the cell. Furthermore, isotonicity must now be considered a relatively inexact term because the ionic equilibrium of each cell is determined by its previous ionic environment, by its metabolic state, and by the environmental temperature, to say nothing of many factors that we can only conjecture. Bacteria, whether wild or captive, are not blessed with the relatively well-regulated "*milieu intérieur*" of the mammals!

Bacterial cells containing chromatin arranged as an axial filament are found not only in older cultures but also in small numbers in young cultures. Delaporte (11) considered that all other conformations were derangements of the axial filament form. On the basis of the experiments here reported

we must question the importance that she ascribed to the axial filament and can suggest that individual cells may be metabolically inept in an otherwise normal population. The process of vital staining ("*coloration sublethale*" in earlier papers) with brilliant cresyl blue, on which she relied as an index of the natural configuration, must be considered as suspect. Cytoplasmic inclusions do modify configurations in some species, as she points out, and this has been confirmed (32).

We must emphasize that the aggregation of chromatin induced in young growing cells involves not only a single nuclear structure but can proceed to the *fusion* of two or more apparently separate structures within the same cell. These fusions can be reversed in most instances and the chromatin configurations that result are indistinguishable in all ways from those of untreated control cells. Aggregation and fusion are inhibited in a salt-deficient environment. A critical view must be taken, therefore, of the interpretation by DeLamater (9, 10) that antibiotics act in bacteria as mitotic poisons; it seems more likely that the observed effects are part of a more general metabolic and physicochemical phenomenon induced by toxic substances.

In conclusion, it is proposed that chromatin aggregation is a function of the interaction of cations and negatively charged chromatin units. And the response of the bacterial chromatin, acting as an internal indicator of ionic equilibrium, to various environmental circumstances depends upon the ionic environment and the integrity of metabolic functions essential to normal ion regulation. Using this as a working hypothesis, studies on nuclear structure in phage-infected cells and during fixation have been undertaken. These results will be reported in further publications.

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COMMENTS ON THE CLASSIFICATION OF THE ORDER RICKETTSIALES[1]

By Cornelius B. Philip

Abstract

The classification of the Rickettsiales is discussed for taxa above species. A new Class, Microtatobiotes, meaning smallest life, is proposed to include Orders Rickettsiales and Virales. Other new proposals are: Tribe Wolbachiae under Rickettsiales including two new genera *Symbiotes* (genotype *Rickettsia lectularia* Arkwright *et al.*) and *Rickettsiella* (genotype *Coxiella popillae* Dutky and Gooden). Extensive new synonymy is provided in genera and species under *Rickettsia, Coxiella, Cowdria, Miyagawanella,* and *Wolbachia.*

Introduction

The present discussion is offered in anticipation of the need for revision of Supplement I, the Rickettsiales, as it was treated in the sixth edition of *Bergey's Manual of Determinative Bacteriology* (Williams and Wilkins, 1948), and to reduce needed explanation and footnotes for certain nomenclatural changes to be incorporated in that Order in the forthcoming seventh edition.

Order Rickettsiales Buchanan and Buchanan

(Bacteriology, 4th ed., New York, 1938, 49; emend. Gieszcyzkiewicz, Bull. Intern. Acad. Polon. Sci.; Classe Math. Nat., B (1), 1939, 9–30).

The original proposal by Buchanan and Buchanan included mainly what is now treated in the Family Rickettsiaceae, and the content was later expanded by Gieszcyzkiewicz to include the present conception of the Order.

There is need for a higher taxon (Class) which will include the Orders Rickettsiales and Virales on a categorical level with the Class Schizomycetes, the bacteria proper. Zhdanov (35), also see Breed and Petraitis (2), even went a category higher in proposing a Phylum Vira composed of three Classes, I. Protophytoviralia, II. Phytoviralia, and III. Zooviralia, for essentially the same subject matter. While this has some merit, its adoption would apparently require an equivalent elevation for the bacteria proper, which hardly seems necessary or appropriate. Zhdanov's use of the virus stem also gives an inadequate idea of the content of his categories by overemphasizing the viral and obscuring the rickettsial and related elements included in the group. It is doubtful if Zhdanov's higher categories will be widely accepted. For a similar reason the earlier proposal of Class Vira by Holmes (11) for the viruses only would not seem appropriate for the more inclusive concept.

[1]*Manuscript received September 27, 1955.*
Contribution from the U.S. Department of Health, Education, and Welfare; Public Health Service, National Institutes of Health; National Microbiological Institute, Rocky Mountain Laboratory, Hamilton, Montana.

Microtatobiotes (meaning smallest life) is here proposed as a more appropriate and comprehensive Class name to include the accepted Orders Rickettsiales and Virales.

The ordinal name *Rickettsiales* has been criticized as not broad enough to acknowledge the varied composition in the Order, especially since it is based on the most junior of the type genera of the three contained families. However, the name now appears to be well established, and its shortcomings are overlooked by those familiar with its coverage.

The content of the three families will remain essentially the same in the new edition, namely, I. Rickettsiaceae Pinkerton; II. Family nov.[2] to be substituted for "Chlamydozoaceae Moshkovskiy" (contents prepared as previously by G. W. Rake, though Coles (4) has questioned the need for separation of this group from the first family); and III. Bartonellaceae Strong *et al.* (prepared by D. Weinman). The second group will thus be moved nearer the more closely related first family than in the sixth edition. A section on *Anaplasma* prepared by W. O. Neitz will be added either as an additional family or as an appendix to the Bartonellaceae, and B. M. Honigberg will revise Kirby's previous appendix on intracellular parasites of Protozoa.

There are still many who conservatively believe that a large genus *Rickettsia sens. lat.* is preferable to the splitting of elements divergent from the genotype, *R. prowazekii*, into smaller genera; Weyer is among those who recently have urged more conservatism in classification of the rickettsiae. There are others, fortunately few, who have proposed generic names almost to the point where the difference between a genus and a species disappears, and classification becomes an end in itself rather than a utilitarian device for reference purposes. A middle course has been the aim in present revision beneath the family category and the introduction of tribal and subgeneric taxa has seemed the best answer to this dilemma in the Family Rickettsiaceae.

Comments on Tribes

Two of the proposed tribes were previously anticipated (Philip (26)), I. Rickettsieae (typographically misspelled Rickettsiaceae) and II. Ehrlichieae. To these a third is now added, III. Wolbachieae.

The Rickettsieae will include the genera *Rickettsia* da Rocha Lima and *Coxiella* Philip.

The Ehrlichieae will include *Ehrlichia* Moshkovskiy, *Cowdria* Moshkovskiy, and *Neorickettsia* Philip, Hadlow, and Hughes. No further information on affinities has been forthcoming since the original description of *Rickettsia*

[2] *While this manuscript was in press, Dr. R. E. Buchanan checked the original references for the writer on the type genus, Chlamydozoon Prowazek, 1907, and found that Moshkovskiy's family is erroneously based on the later described and assigned genotype species, Rickettsia trachomatis Foley and Parrot, 1937, whereas the genus is monotypic for the originally validly published Chlamydozoon bombycis Prowazek 1907 (see Intern. Bull. Bacteriol. Nomen. Taxon. 5 : 121-128. 1955).*

(*Donatienella*) *delpyi* Rousselot (27) isolated from an Iranian gerbil. Giroud *et al.* (8) raised *Donatienella* to full generic status for the agents of the animal rickettsioses here placed in *Ehrlichia*.

The Wolbachieae will include *Wolbachia* Hertig and two new genera, *Symbiotes* and *Rickettsiella*, described below.

One of the questions most often discussed or implied in proffered reassignment by contemporary authors is what species, subsequently proposed and mostly assigned to *Rickettsia sens. lat.*, are really congeneric with the genotype species, *R. prowazekii* da Rocha Lima. Again a middle-of-the-road viewpoint appears preferable for the present revision. This is best implemented by recognition of subgenera, though this still will not account for all species that have been described as *Rickettsia* spp., too often because of a lack of a more precise allocation pending adequate elucidation. This has resulted in the generic name becoming almost a dumping ground for any species that are arthropod-borne, intracellular, and/or noncultivable on artifical media, and sometimes pathogenic for vertebrates with or without an arthropod vector. Under these conditions, it is apparent why earnest opinions of investigators have often been at variance. The fact is too often lost sight of that Nature evolved but did not classify; man did that for his own convenience, though sometimes it seems more to his inconvenience.

Yet there is no denying that the genus *Rickettsia sens. lat.* still remains the most popular assignment for newly described species of apparent relationship for which information is inadequate for more exact placement. Some of these difficulties can be resolved, it is believed, in the new edition of the Manual by assignment of the inhabitants of arthropods of no known or probable pathogenicity for vertebrates, to the genus *Wolbachia* in the new Tribe Wolbachieae, thus narrowing the genus *Rickettsia* to a more utilitarian taxonomic unit which is in keeping with the restricted concept of species congeneracy with *R. prowazekii*.

Each of the following which were originally proposed as genera was reduced by Philip (24, 26) to the subgeneric level (in addition to the typical subgenus *Rickettsia*): *Dermacentroxenus* Wolbach, *Zinssera* Macchiavello, and *Rochalimae* Macchiavello.

Disregard of International Rules has resulted in the following new synonymy:

(1) *Rickettsia* subgen. *Ixodoxenus* Zhdanov (35) equals *Rickettsia* subgen. *Dermacentroxenus* Wolbach. Zhdanov included four species in his subgenus without type designation, and the first is herewith selected as subgenotype species, namely, *Ixodoxenus ricksettsi* Zhdanov (= *R.* (*D.*) *rickettsii* (Wolbach) Brumpt). Zhdanov's (35) varieties, *R. rickettsi occidentalis*, *R. r. orientalis*, and *R. r. brasiliensis*, as well as *Rickettsia colombiensis* Vientemillas, 1944, are insufficiently differentiated from *R. rickettsii* to be recognized as more than synonymous. Zhdanov (35), as have occasional authors before him, repeatedly overlooked the fact that varieties (and subgenera) must include a typical form retaining the parent name (Rule 5), e.g., *R.* (*D.*) *rickettsii* var. *rickettsii*, which

in this instance would have replaced his *R. r.* var. *occidentalis* had Zhdanov's varieties been valid. His *R. r.* variety *brasiliensis*, moreover, would have been antedated by *R. brasiliensis* Monteiro (18) (= *R. rickettsii*) while his *R. r.* var. *orientalis* is a homonym of *R. orientalis* Nagayo *et al.* (21).

Furthermore, Zhdanov proposes two varieties of *Rickettsia* (*Ixodoxenus*) *conorii* Brumpt which are also synonyms; his *R. c.* var. *mediterraneus* cannot replace the typical form, and *R. c.* var. *africanus* is antedated by *R. megawi* var. *pijperi* Mason and Alexander (16), though the agent of South African tick-bite fever is not considered at present as even subspecifically different from that of typical fièvre boutonneuse.

Responsibility for the prior name of the etiologic agent of Siberian tick typhus is still obscure because of unavailability of several pertinent references. Ogata (23) introduced an apparent *nomen nudum*, *Rickettsia nuttalli*, crediting it to Pavlovskiy 1940[3] probably on the basis of the latter's reference to one of the tick vectors, *Dermacentor nuttalli* in Siberia. Krassilnikov (13) introduced the name *Rickettsia* (*Dermacentroxenus*) *sibiricus* crediting it to Moshkovskiy on the basis of "*Dermacentroxenus* sp." Moshkovskiy (20). Zhdanov (35) proposes the new combination *Rickettsia* (*Ixodoxenus*) *sibiricus* for *Dermacentroxenus sibiricus* Zdrodovskiy (33). Zdrodovskiy and Golinevitch (34) list an earlier *D. sibiricus* allegedly described by them in 1948 (probably published 1949). Considering that a strain of *R.* (*Dermacentroxenus*) *conorii*, which was not considered sufficiently different to warrant a name (Philip (25)), has been identified from ticks as far east as Kashmir, India, it appears not unlikely that the agent of Siberian tick-borne rickettsiosis is the same or a variant transmitted by indigenous tick vectors. Until more studies are made than those of Korshunova (12) on the cross-relationships of the Siberian agents with known typhus-like *Rickettsia* spp., it will be difficult to classify the Siberian rickettsiae with certainty. Of five strains received in this country through diplomatic channels by the writer in 1946–47, the only one that carried the label "tick-borne fever" was never reactivated. The other four strains (labelled when received "XON," "DII," "epidemical typhus," and "louse-borne typhus," the last two identified in correspondence as strains "G" and "B" respectively) proved to be related to *R. prowazekii* as reported by Crocker *et al.* (6) and concomitantly confirmed at the Rocky Mountain Laboratory (unpublished). Unfortunately, the idea that Siberian tick typhus and louse-borne typhus are closely related has resulted, e.g., Smith *et al.* (30, p. 646), but this confusion cannot be resolved until *bona fide* strains from Siberian tick sources become available for comparison.

(2) *Rickettsia* subgen. *Gamasoxenus* Zhdanov (35) genotype by present designation and only species *R.* (*G.*) *muris* Zhdanov (35) (= *R. akari* Huebner *et al.*). This was invalidly (Rule 23) substituted for *Acaroxenus* Zhdanov and Korenblit (36) genotype by present designation and presumed

[3] *After failing in attempts to locate such a reference, the writer, in personal conference with Pavlovskiy in Copenhagen, August 1953, learned that the latter disclaimed ever proposing a Latin name for this agent, which statement is corroborated by lack of any subsequent reference in recent Russian publications.*

only species *A. varioloidis* Z. & K. (= *R. akari* Huebner *et al.*). Both subgenera equal *Rickettsia* subgenus *Dermacentroxenus* (Wolbach) Brumpt. This substitution by Zhdanov for his own earlier binomials is not admissible under the Rules. However, should a future reviewer decide that the agent of mite-borne rickettsialpox warrants separate subgeneric recognition, then the name *Acaroxenus* is available.

(3) Genus *Trombidoxenus* Zhdanov and Korenblit (36) equals subgenus *Zinssera* Macchiavello (15) (Philip (26)). Genotype species of the former herewith designated as *T. orientalis* Zhdanov (= *R.* (*Z.*) *tsutsugamushi* (Hayashi) Ogata). Since Philip (26) cited evidence supporting subgeneric recognition of *Zinssera*, Weyer (32) has additionally demonstrated the peculiar difficulty of cultivating *R. tsutsugamushi* in body lice and other arthropods compared to other rickettsiae. Krassilnikov (13) wrongly placed *R. orientalis* in subgenus *Dermacentroxenus* apparently because it is acarid-borne.

(4) Megaw (17) made the following provisional proposal "...a subgeneric name for the organism of trench fever...might be *Fossilis quintana* but it is hoped that this complication might be avoided..." without offering any further light on this name. This provisional name is therefore considered under Rule 12 not to antedate the validly proposed *Rickettsia* subgen. *Rochalimae* Macchiavello 1947 (Philip (26)). *Welhynia* [sic] Zhdanov and Korenblit (36) is a synonym; subgenotype herewith designated as the first of two contained species, *W. quintanae* Z. & K. (36) (= *R.* (*R-1.*) *quintana* Schmincke (28)).

(5) "*Coxiella diaporica* comb. nov. (Derrick 1937)" is another invalid rearrangement by Zhdanov (35) which still remains a synonym of the prior name *C. burnetii* (Derrick) Philip, which species Zhdanov listed as a synonym of the former. Zhdanov and Korenblit (36) had referred to this agent as "*Cexiella diaparica.*"

(6) *Kurlovia* Zhdanov (35) was invalidly erected to contain *Cowdria* Moshkovskiy (20) and *Ehrlichia* Moshkovskiy (19) both of which prior names Zhdanov invalidly degraded to subgeneric status. The first contained species, *K.* (*Cowdria*) *ruminantium* (Cowdry) Zhdanov, is herewith designated as genotype and *Kurlovia* becomes a synonym of genus *Cowdria*. Here again Zhdanov ignored the necessity (Rule 5) for a typical subgenus *Kurlovia* for acceptable nomenclatural practice. Synonyms of *Cowdria* also are *Nicollea* Macchiavello (15) (same genotype species) and possibly *Rickettsia* subgen. *Donatienella* Rousselot (27) (genotype and only species *R.* (*D.*) *delpyi* Rousselot) though the latter may be closer to the agents assigned to *Ehrlichia* as interpreted by Giroud *et al.* (8).

(7) *Rickettsiaformis* Zhdanov and Korenblit (36) (genotype by present designation and last of 16 contained species, *R. lymphogranulomatis* Z. & K. (36)) (= *Miyagawanella lymphogranulomatosis* Brumpt) which genus thus becomes a synonym of *Miyagawanella* Brumpt. The first included species is the congeneric *Rickettsiaformis psittacosis* Zhdanov and Korenblit (36) (= *Miyagawanella psittacii* (Lillie) Rake).

(8) *Cowdryia* Macchiavello (15) (genotype species *Rickettsia lectularia* Arkwright *et al.*) appears to be homonymous as a slight orthographic variant of the prior *Cowdria* Moshkovskiy 1945 (Rules 25 and 27). *Cowdryia pulex* Macchiavello (15) was invalidly proposed with nine varieties including the prior *R. ctenocephali* Sikora (29) which he designates as "type variety"; two of his varieties, *C. ctenocephalocanis* and *C. irritans*, are credited to Cowdry (5) who described but did not name them, so that Macchiavello (15) becomes the author. *C. pulex* Macchiavello, therefore, is a synonym of *Wolbachia ctenocephali* (Sikora) Phil. If the character of different flea hosts without other differentiating characters which Macchiavello uses for his eight other varieties is substantiated by further study, the names would become varieties of *W. ctenocephali*, or separate species.

Tribe III Wolbachieae nov.

Includes species which were assigned to *Wolbachia* and *Rickettsia sens. lat.*, and which are rickettsialike in growth, morphologic properties, and staining properties, and are mostly intracellular symbiotes or parasites of suitable species of arthropods. Pleomorphism is especially characteristic. They may be restricted to certain tissues or mycetomes, or generally distributed in the bodies of the hosts. Fatality among the latter may be nil to 100% for different species of parasites. No pathogenicity for vertebrates has been reported, though this is disputed in the case of the sheep ked organism, *W. melophagi*. Most species have resisted cultivation in any media other than the living host, but one, *W. melophagi*, has been reported as being cultivable on cell-free media. The systematic status of *R. rochalimae* is still uncertain but growth anaerobically on nonliving media has been claimed.

Characterization has not been as adequate for many species now assigned here as in the case of pathogens of vertebrates. It is the writer's opinion that systematic progress in the family will be advanced by broadening the concept of the genus *Wolbachia*, just as has been done with *Rickettsia*. The particular type of pleomorphism described for the genotype species, *W. pipientis* Hertig (9), is thus recognized as of specific rather than generic value. Other species can then be assigned to this genus.

Napier (22) also recognized this need when he proposed the full family Wolbachaceae "for nonpathogenic, and potentially extra-cellular organisms" under his superfamily Rickettsoidea (with invalid zoological suffix that is too close to the accredited bacteriological ending for subfamily, -oideae). His character "nonpathogenic" referred to vertebrate hosts, and his concept of extra-cellular was obviously in relation to species now known to be synonyms of *R. quintana*. The family level appears too high to separate *Wolbachia* from *Rickettsia*. However, differentiation of this group is warranted above the genus, since there is now more than one genus in each of the two major groups tabulated by Steinhaus (31, pp. 266–7) on the basis of pathogenicity for vertebrates. There probably will be more genera as the Wolbachieae become better known.

Genus I. *Symbiotes* gen. nov. Rickettsialike, pleomorphic organisms living chiefly intracellularly in arthropod tissues, and approaching most nearly the true mutualistic or commensalistic relationship to their hosts, as evidenced by the development of special organs, or mycetomes, in the host though no species of the Rickettsiales is yet known to be confined to such a location.

Genotype species, *Symbiotes lectularia* (Arkwright, Atkin, and Bacot (1)) comb. nov. in *Cimex lectularia*, the common bedbug.

Cowdryia Macchiavello (15) was previously proposed with this same genotype species, but the name is invalid by homonymy with *Cowdria* Moshkovskiy (20) as discussed earlier.

The parasite–host relationship of this species has approached such mutualism that Steinhaus (31) has remarked "it seems probable that every bedbug harbors the organism" which occurs not only in paired mycetomes, but in other tissues as well, including embryonic. Swelling of infected cells in the Malpighian tubules is the only reported effect on the host though no beneficial relationship has been postulated. It appears probable that motility of some of the filamentous forms reported by Hertig and Wolbach (10) and noncultivability in cell-free media are specific rather than restrictive generic characters. It also appears likely that this genus represents a very long association with the host in view of the approach to mutualism that has been achieved, so that the genus is listed first in the subfamily. Unfortunately, filterability has not been tested.

Genus II. *Wolbachia* will have transferred to it from *Rickettsia* the following species: *W. ctenocephali* (Sikora) comb. nov., *W. culicis* (Brumpt) comb. nov., *W. dermacentrophila* (Steinhaus) comb. nov., *W. linognathi* (Hindle) comb. nov., *W. melophagi* (Noller) comb. nov., *W. pulex* (Macchiavello) comb. nov. and 8 vars., *W. sericea* (Giroud and Martin) comb. nov., and *W. trichodectae* (Hindle) comb. nov. *Ixodi-symbiotes* Brumpt (3) was proposed "pour les nombreux symbiotes specifiques des ixodides" without assignment of a species, and the name remains a *nomen nudum*.

Genus III. *Rickettsiella* gen. nov. Minute, intracellular, rickettsialike organisms which are pathogenic for certain insect larvae but not known or likely to be for any vertebrates; filterable, associated with intracellular, crystalline inclusions, and reported to infect cell nuclei. The diminutive ending is appropriate for the generic name of this filterable parasite which was related to and originally described in the genus *Coxiella* on this basis.

Genotype species, *Rickettsiella popilliae* (Dutky and Gooden (7)) comb. nov., the etiologic agent of blue disease of beetle larvae.

This species has been isolated from larvae of the Japanese beetle (*Popillia japonica*) and of two species of June beetles (*Phyllophaga*). Infection is first evidenced by a bluish tinge of the fat body, infected cells of which appear intensely blue by dark-field examination. The discoloration in the larvae becomes more pronounced as the disease progresses to death. The organisms are kidney-shaped rods of $0.2 \times 0.6\ \mu$ as seen under the electron microscope and are filterable in quantity through a fine porosity (13-lb. Mandler) filter

which was the reason for original assignment to the genus *Coxiella*. Masses of organisms and crystalline inclusions of 1–3 μ in size are seen to issue from ruptured, infected cells in microscopic preparations. Cultivation not reported other than experimental propagation by feeding and subinoculation of healthy hosts.

Filterability alone is not considered sufficient to make this congeneric with *Coxiella burnetii*, the agent of human Q fever. Preparations from ticks and mealworms infected with the latter have never shown evidence of associated crystalline inclusions. There are also fundamental differences in host and host reactions.

Rickettsia melolonthae Krieg (14) is also assignable to *Rickettsiella*, and indeed may be only a variety or synonym of the above based on similarities in morphology, filterability, pathogenicity for beetle larvae, and association with microscopic crystals.

Filtration and electron microscope studies are obviously desirable for species assigned to *Wolbachia* above.

Discussion

A comparison of the taxa above the rank of species in the group in Supplement I of the sixth edition of Bergey's Manual to those proposed for the forthcoming (seventh) edition is outlined below. It is expected that the Orders Rickettsiales and Virales, treated as Supplements previously, will now be transferred to the main text and assigned to Class Microtatobiotes. Dr. R. S. Breed, in another paper in this issue, has discussed the need for this action.

	Sixth ed. (1948)	Seventh ed. (in press)
Order:	Rickettsiales	Rickettsiales
Family:	Rickettsiaceae	Rickettsiaceae
Tribe:	None	Rickettsieae
Genus:	1. *Rickettsia* *Subgenera* (none)	1. *Rickettsia* *Subgenera: Rickettsia, Dermacentroxenus, Zinssera,* and *Rochalimaea* (emend.)
	2. *Coxiella*	2. *Coxiella*
	3. *Cowdria*	
Tribe:	None	Ehrlichieae
		1. *Ehrlichia*
		2. *Cowdria*
		3. *Neorickettsia*

Tribe:	None	Wolbachieae 1. *Wolbachia* 2. *Symbiotes* gen. nov. 3. *Rickettsiella* gen. nov.
Family:	Chlamydozoaceae	fam. nov. (by Rake)
Genus:	1. *Chlamydozoon* 2. *Miyagawanella* 3. *Colesiota*	1. gen. nov. 2. *Miyagawanella* 3. *Colesiota* 4. gen. nov. 5. gen. nov.
Family:	Bartonellaceae	Bartonellaceae (by Weinman)
Genus:	1. *Bartonella* 2. *Haemobartonella* 3. *Grahamella* 4. *Eperythrozoon*	1. *Bartonella* 2. *Haemobartonella* 3. *Grahamella* 4. *Eperythrozoon*
Family:	None	Anaplasmataceae 1. *Anaplasma* (by Neitz)
Appendix	[Intracellular parasites of Protozoa] (by Konigberg)	

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L'IMMUNITÉ DANS LES INFECTIONS STAPHYLOCOCCIQUES

1. ACTION DE L'ANTITOXINE CONTRE UNE INFECTION BACTÉRIENNE EXPÉRIMENTALE[1]

A. FRAPPIER ET S. SONEA

Abstract

Using the intracerebral route in mice immunized with commercial antitoxin, complete or significant protection was obtained for the first time at will against lethal doses of living staphylococci. The protection was effective against the five staphylococcus strains used. The mechanism of this experimental infection was partially studied in normal and in immunized mice by counting at variable intervals the living bacteria at the inoculation point (brain) and in the blood. A moderate bacteraemia was present immediately after the inoculation, decreased later to few bacteria per drop of blood, and eventually disappeared. This bacteraemia was not influenced by the presence of the antitoxin. At the inoculation point, a marked decrease in the number of staphylococci was evident immediately after the injection of the bacteria, and one hour later. The antitoxin had no appreciable effect on this phenomenon. The local multiplication following the above mentioned decrease in the number of staphylococci was moderate and was not checked by the antitoxin. Mortality in unprotected animals seems to be caused by staphylococcal toxins liberated in greater quantity by the increased number of living staphylococci found about the fourth hour after inoculation.

Introduction

Une difficulté rencontrée depuis longtemps dans l'étude de l'immunité antistaphylococcique, réside dans le fait qu'on n'a pas réussi jusqu'ici à protéger à volonté les animaux de laboratoire contre une dose mortelle, non pas de toxine, mais de *staphylocoques vivants*. C'est la raison principale pour laquelle on ne peut déterminer le rôle que peuvent jouer réellement dans l'infection bactérienne naturelle les toxines (*alpha*, *beta* ou autre) ou les produits pro-infectieux (coagulase, polysaccharides de surface, hyaluronidase, etc.).

Chez des lapins inoculés avec des staphylocoques vivants, à l'exception de quelques cas exceptionnels, imprévisibles, le seul résultat de l'immunisation active ou passive est une *diminution temporaire* de la gravité des lésions avec prolongation de la survie (1, 2, 3, 4, 5, 7, 14, 15, 17, 20, 22, 23, 24, et 25). Pour ce qui est de la souris immunisée, nous connaissons seulement deux publications où l'on rapporte avoir obtenu une protection partielle contre des doses mortelles de staphylocoques vivants administrés dans les deux cas par voie intrapéritonéale (16, 23). Nous n'avons pu confirmer ces résultats. Tout récemment, on a pu réussir une diminution partielle de la mortalité chez des

[1]*Manuscrit reçu le 18 octobre 1955.*
Une partie du sujet a été communiquée au Congrès annuel de la Société Canadienne des Microbiologistes, à Winnipeg, juin 1955.
Contribution du Département de Bactériologie de la Faculté de médecine et de l'Institut de Microbiologie et d'Hygiène de l'Université de Montréal. Travail effectué à l'aide d'un octroi des Fondations Joseph Rhéaume.

poulets de huit semaines immunisés activement et inoculés par voie intraveineuse avec des staphylocoques conférant une infection mortelle à 80% des animaux non immunisés (26).

Dans le présent travail, nous avons, au moyen des nouvelles méthodes d'infection staphylococcique expérimentale mises au point dans nos laboratoires, réalisé des conditions dans lesquelles il est possible de mettre en évidence chez les animaux immunisés un état de résistance appréciable contre une épreuve au moyen de bactéries vivantes.

Après avoir essayé la méthode d'inoculation par voie intrapéritonéale chez la souris et celle de l'inoculation de l'embryon de poulet de 19 jours, nous nous sommes, pour le moment, arrêtés à l'inoculation intracérébrale de la souris. Avec cette méthode, le nombre efficace de bactéries à inoculer est raisonnable, il existe une multiplication appréciable des bactéries précédant la mort des animaux infectés et, pour une même souche, les taux de mortalité obtenus sont constants et proportionnels aux doses utilisées.

Pour déterminer le seul rôle de l'immunité humorale, avec réduction au minimum de la participation tissulaire, nous nous sommes adressés à l'immunisation passive.

Matériel et Méthodes

Les souches de staphylocoques utilisées sont toutes hémolytiques, à coagulase positive et à mannitol positif. La souche Wood 46 est surtout connue à cause de sa propriété de produire beaucoup d'hémolysine *alpha* et peu d'autres hémolysines. Les souches 641 et A8 ont un pouvoir pathogène élevé pour la plupart des animaux de laboratoire. Les souches 438 et 439 proviennent d'infections humaines récentes. A la lumière des travaux d'Elek et Levy sur les hémolysines (6), nous avons considéré inutile de procéder à une détermination quantitative des hémolysines produites par ces souches. L'inoculum est constitué dans la plupart des expériences par 0.03 ml. de culture staphylococcique totale de 18 à 24 h., en bouillon ordinaire, suivant la méthode d'infection de Panisset et Dobija (21). Nous avons adopté récemment un inoculum de staphylocoques prélevés sur culture de 18 h. sur gélose, lavés une fois et remis en suspension avec de l'eau salée pour donner selon les souches une transmission de 74% à 85% au spectrophotomètre Coleman junior, longueur d'onde 420 (0.03 ml. par souris).

Les animaux utilisés sont des souris blanches de 18 ou de 19 g., grosseur qui permet une inoculation intracérébrale facile. Nous avons employé une antitoxine commerciale* contenant 10,000 unités dans 12 ml. Comme toutes les antitoxines, celle-ci contient d'autres anticorps à côté des anti-hémolysines *alpha*, mais en moindre proportion (6, 19).

L'inoculation intracérébrale des staphylocoques est faite suivant la méthode déjà mentionnée (21). Les anticorps sont administrés par voie intrapéritonéale à des intervalles de temps variables d'une expérience à l'autre. On relève les derniers résultats 24 h. après l'inoculation des staphylocoques.

**Antitoxine staphylococcique de Connaught Medical Research Laboratories.*

Pour étudier le mécanisme de l'immunité antistaphylococcique, nous avons suivi dans le cerveau et dans le sang du coeur des souris infectées, immunisées et non immunisées, le devenir des staphylocoques inoculés. Le cerveau d'une souris inoculée est prélevé aseptiquement et broyé dans un mortier en présence de 10 ml. d'eau salée. Des dilutions successives de la suspension ainsi obtenue sont effectuées et les bactéries vivantes sont comptées en ensemençant 0.1 ml. de chaque dilution dans des géloses fondues portées ensuite en boîtes de petri. Parallèlement, une ou deux gouttes du sang du coeur des mêmes animaux ont été ensemencées de la même façon, sans dilution préalable.

Résultats

Dans une première expérience avec la souche Wood 46, nous avons obtenu un important degré de protection avec 0.5 ml. d'antitoxine non diluée (exp. A, tableau I). Dans une deuxième expérience avec la même souche (exp. B, tableau I), les souris ont été protégées par 1 ml. d'antitoxine diluée à ½ mais elles n'ont pas été protégées par 0.5 cc. d'une dilution au quart de la même antitoxine.

Dans une troisième expérience (exp. C, tableau I) nous avons voulu déterminer quel était l'effet de trois dilutions d'antitoxine sur une infection moins sévère. L'inoculum est constitué de 0.03 ml. d'une dilution au dixième de la culture totale, ce qui donne d'habitude une mortalité d'environ 50% pour la souche Wood 46 utilisée dans cette expérience. Pour un inoculum moins fort, on obtient une protection accrue.

TABLEAU I

ACTION *in vivo* DE L'ANTITOXINE STAPHYLOCOCCIQUE CONTRE LA SOUCHE WOOD 46

Expérience	Substance administrée quelques minutes avant l'inoculation des staphylocoques	Mortalité provoquée
A	Antitoxine staphylococcique, 0.5 ml. non diluée	1/20
	Témoins: 0.5 ml. eau salée	16/20
B	1 ml. d'antitoxine staphylococcique diluée à 1/2	3/10
	0.5 ml. antitoxine staphylococcique diluée à 1/4	10/10
	Témoins: 1 ml. antitoxine diphtérique diluée à 1/2	10/10
	Témoins: 0.5 ml. antitoxine diphtérique diluée à 1/4	10/10
C	0.5 ml. antitoxine staphylococcique diluée au 1/10	0/10
	0.5 ml. antitoxine staphylococcique diluée au 1/100	1/10
	0.5 ml. antitoxine staphylococcique diluée au 1/1000	3/10
	Témoins: 0.5 ml. eau salée	6/10

TABLEAU II

ACTION *in vivo* DE L'ANTITOXINE STAPHYLOCOCCIQUE CONTRE DIVERSES SOUCHES DE STAPHYLOCOQUES

Expérience	Substances administrées	Mortalité provoquée par diverses souches				
		Wood 46	A8	641	438	439
A	Antitoxine staphylococcique diluée à 1/2	2/10	1/10	5/10		
	Témoins: antitoxine diphtérique diluée à 1/2	9/10	9/10	8/10		
B	Antitoxine staphylococcique diluée à 1/2	4/10	0/10	0/10	0/10	0/10
	Témoins: antitoxine diphtérique diluée à 1/2	10/10	8/10	9/10	10/10	9/10
C	Antitoxine staphylococcique diluée à 1/2	0/10	2/10	4/10	2/10	5/10
	Témoins: antitoxine diphtérique diluée à 1/2	9/10	6/10	5/10	8/10	5/10

Dans trois autres expériences avec diverses souches, la dose de 0.5 ml. d'une dilution à ½ d'antitoxine a protégé contre une infection mortelle pour les témoins (tableau II). On remarque une variation dans les chiffres d'une expérience à l'autre due probablement à l'utilisation de l'inoculum empirique constitué par la culture totale.

Dans trois expériences entreprises pour déterminer le devenir des staphylocoques inoculés en doses mortelles chez des souris non immunisées, nous avons d'abord comparé deux souches entre-elles, Wood 46 et A8. Pour des fins pratiques, des groupes de deux ou de trois souris seulement étaient utilisés pour chaque souche et chaque intervalle de temps. Le tableau III reproduit les résultats de ces trois expériences effectuées avec un inoculum contenant 0.03 ml. d'une culture totale en bouillon ordinaire.

TABLEAU III

NOMBRE DE COLONIES OBTENUES À PARTIR DE SOURIS NORMALES, À INTERVALLES VARIABLES APRÈS L'INOCULATION INTRACÉRÉBRALE DE CULTURE EN BOUILLON DE DEUX SOUCHES DE STAPHYLOCOQUE. INOCULUM DE 400 BACTÉRIES POUR DILUTION 10^{-5}

	Moyenne expériences cerveau dil. 10^{-5}		Expérience 1 3 souris par souche, cerveau dil. 10^{-5}		Expérience 2 2 souris par souche, cerveau dil. 10^{-4}		Expérience 3 2 souris par souche			
							Cerveau dil. 10^{-5}		Sang 1 goutte non diluée	
	Wood 46	A8	Wood 46	A8	Wood 46	A8	Wood 46	A8	Wood 46	A8
Immédiatement	74	8.5	160 5 80	12 11 14	44 132	11 2	38 60	5 5	680 18	292 14
2 h. après	444	164	—		—		624 264	184 144	10 3	12 4
3 h. après	850	422	—		656 1045	500 344	—		—	
4 h. après	528	1048	—		—		320 736	1160 936	4 1	88 11
6 h. après	m	1616	—		m m	>3500 >3500	m m	1616 776	m m	52 3

m = souris mortes vers la cinquième heure.

Dans les cerveaux prélevés immédiatement après l'injection, et pour des inoculums pratiquement équivalents, constitués d'une culture totale en bouillon, on trouve en moyenne huit fois plus de staphylocoques de la souche Wood que de staphylocoques de la souche A8. Plus tard, vers la quatrième heure, la souche A8 semble se multiplier plus rapidement ou résister mieux que la souche Wood 46 dont la multiplication reste stationnaire ou diminue, ce qui renverse le rapport antécédent.

Dans ces expériences, on n'a pas continué les numérations pour la souche Wood 46 après quatre heures car les souris inoculées avec la culture totale de cette souche sont toutes mortes vers la cinquième heure tandis que les souris injectées avec la souche A8 malgré l'augmentation évidente du nombre de bactéries sont mortes seulement quelques heures plus tard (9–19 h. après l'inoculation).

Dans le sang du coeur, le plus grand nombre de bactéries apparaît dans les premières minutes après l'injection des staphylocoques. Par la suite, le nombre y reste très faible (tableau III).

Dans le tableau IV, nous avons reproduit les résultats d'une expérience qui ressemble aux trois précédentes, mais effectuée non plus avec un inoculum contenant une culture liquide totale mais avec une suspension équivalente de bactéries lavées. En plus, au lieu de faire la numération bactérienne sur chaque cerveau, ce qui limiterait les groupes à deux animaux chacun, nous avons broyé ensemble les cerveaux de chaque groupe de quatre souris mises en expérience et effectué la numération sur la suspension de ce broyat dans 10 ml. d'eau salée. L'évolution du nombre de colonies est parallèle à celle que l'on a observée dans les expériences précédentes, mais il y a légère prolongation de la survie des animaux. Les souris sont en effet mortes un peu après la sixième heure, sous l'effet de la souche Wood 46, tandis qu'avec l'inoculum contenant la culture totale, la mort se passe d'habitude vers la cinquième heure après l'inoculation.

Dans le tableau V, nous rapportons l'évolution du nombre de colonies provenant de souris immunisées et non immunisées, inoculées avec la souche Wood 46. Nous avons utilisé un inoculum fort (transmission 74% au spectrophotomètre) dans l'expérience A et un inoculum plus réduit (transmission 85%) pour l'expérience B. On remarque que les staphylocoques des témoins suivent une évolution semblable à celle des expériences précédentes, tandis que les staphylocoques des souris immunisées et protégées, après une diminution qui semble légèrement plus forte que celle des témoins une heure après l'inoculation, se montrent en nombre plus grand à la quatrième et à la sixième heure.

En général, immédiatement après l'inoculation et même après la première heure, au niveau du cerveau, le nombre de bactéries diminue par rapport au nombre inoculé, chez les animaux immunisés ou non immunisés, mais ce phénomène est constamment plus marqué pour la souche A8 que pour la souche Wood 46, celle-là même qui est réputée très toxique (tableaux III, IV et V). Puis la multiplication démarre et, vers la quatrième heure, on

TABLEAU IV

ÉVOLUTION DU NOMBRE DES COLONIES OBTENUES À PARTIR DE SOURIS INOCULÉES AVEC DES BACTÉRIES LAVÉES PROVENANT DE DEUX SOUCHES DIFFÉRENTES

Inoculum	Sang 1 goutte non diluée 4 souris séparément				Cerveau							
					10^{-4}		10^{-5}		10^{-6}		10^{-7}	
	Wood 46		A8		W. 46	A8	W. 46	A8	W. 46	A8	W. 46	A8
Inoculum	—		—		>3500	>3500	344	400	19	27	4	3
1 h. après	>3500	>3500	>3500	0								
	>3500	1	0	0	1208	394	200	39	10	3	3	0
4 h. après	0	>3500	0	1								
	0	0	0	0	>3500	>3500	444	418	36	27	3	2
6 h. après	0	1	0	0								
	0	0	0	0	>3500	>3500	400	720	88	58	1	3
7 h. après	m		0		m	>3500	m	1096	m	120	m	9

m = souris mortes immédiatement après six heures.

TABLEAU V

ÉVOLUTION DU NOMBRE DES COLONIES OBTENUES À PARTIR DE SOURIS IMMUNISÉES OU NON AVEC L'ANTITOXINE ET INOCULÉES AVEC DES BACTÉRIES LAVÉES SOUCHE WOOD 46

Intervalle après l'inoculation	Expérience A Inoculum contient 444 bactéries pour la dilution 10^{-5}						Expérience B Inoculum contient 117 bactéries pour la dilution 10^{-5}					
	1 goutte sang				4 cerveaux (dilution 10^{-5})		Coeur				4 cerveaux (dilution 10^{-5})	
	I*		T†		I*	T†	I*		T†		I*	T†
1 h.	0	3	14	1	44	60	26	31	0	40	37	73
	1	1	5	1			13	21	0	29		
4 h.	0	1	0	0	1568	360	0	0	1	1	224	152
	0	1	0	0			0	0	1	24		
6 h.	0	0	0	0	3255	280	0	0	0	0	1920	64
	0	0	0	0			0	0	0	0		
24 h.	0	4	m	m	1864	m					3008	m
	0	0	m	m								

**I = résultats chez les animaux immunisés.*
†T = résultats chez les animaux témoins, non immunisés.
m = souris mortes.

retrouve chez les animaux normaux un nombre de bactéries de l'ordre de l'inoculum ou accrus d'environ deux ou trois fois. Si on se réfère au nombre trouvé dans le cerveau immédiatement après l'inoculation (tableau III), l'augmentation vers la quatrième heure varie de 10 fois pour la souche Wood à 20 fois pour la souche A8. Si on se réfère au nombre trouvé une heure après l'inoculation, l'augmentation varie de deux à six fois pour la souche Wood (tableaux IV et V) et de 20 fois environ pour la souche A8 (tableau III). Donc, chez l'animal non immunisé, la souche Wood 46, qui paraissait pendant la première heure avoir une avance sur la souche A8 n'a pas gagné autant de vitesse de multiplication que la souche A8.

La multiplication dans le cerveau ne semble pas diminuée chez les animaux immunisés par l'antitoxine. Le nombre de bactéries augmente d'environ 30 fois entre la première heure et la quatrième dans l'expérience A du tableau V, chez les souris immunisées, c'est-à-dire plus que chez les animaux non immunisés. Dans l'expérience B du tableau V, la multiplication pour la même période est d'environ deux fois pour les animaux témoins et d'environ six fois pour les animaux immunisés.

Six heures après l'inoculation, on trouve chez les animaux immunisés de deux à huit fois plus de bactéries qu'à la quatrième heure (tableau V, expériences A et B). A ce moment, les souris immunisées sont bien portantes mais présentent au moins dix fois plus de bactéries dans leur cerveau que les non immunisées qui sont mourantes. Chez les souris non immunisées, dans les deux heures précédant leur mort, on voit s'arrêter la multiplication des staphylocoques et on observe même une diminution de leur nombre (tableaux III, IV et V).

Discussion

L'infection staphylococcique expérimentale de la souris, provoquée par voie intracérébrale, nous a permis de démontrer qu'il est possible de protéger passivement cet animal au moyen de l'antitoxine commerciale contre des doses mortelles de staphylocoques vivants. La protection s'est avérée efficace contre toutes les souches que nous avons utilisées. Pour mieux comprendre le mécanisme intime de ces phénomènes, nous avons recherché l'évolution du nombre des staphylocoques vivants à l'endroit de l'inoculation et dans le sang des souris infectées, immunisées ou non. A la suite de l'inoculation intracérébrale de la souris, nous avons ainsi constaté au cours des premières minutes chez les animaux immunisés ou non immunisés la présence d'une phase bactériémique très modérée par rapport au nombre de microbes inoculés. Cette bactériémie décroît ensuite rapidement et ne semble pas jouer un rôle important dans le mécanisme de la mort de l'animal. Elle n'est pas non plus influencée par l'antitoxine.

La multiplication bactérienne, dans ces conditions, est donc presque exclusivement locale, au niveau du cerveau. Il y aurait trois phases de cette multiplication à considérer chez *l'animal normal*: une première phase immédiate où le nombre de bactéries est apparemment diminué par rapport à l'inoculum, une seconde (dans les 2–3 h. qui suivent) dans laquelle on voit s'accroître le nombre de bactéries, et une troisième précédant la mort d'une ou de deux heures, au cours de laquelle le nombre de bactéries se stabilise ou décroît légèrement. Chez *l'animal immunisé, par l'antitoxine*, les deux premières phases ne semblent pas subir une modification considérable. Au moment où les témoins non immunisés sont dans la troisième phase, les animaux immunisés présentent une continuation de la multiplication, mais ils survivent grâce à l'action de l'antitoxine.

Si les produits toxiques neutralisables par l'antitoxine commerciale jouaient un rôle précoce dans l'établissement de l'infection, il nous semble que pendant les premières heures, il y aurait constamment un nombre plus faible de staphylocoques chez les animaux immunisés. Il semble que ce n'est qu'au stade final, moment où par suite de l'accroissement du nombre de microbes la libération de produits toxiques arrive à un niveau mortel, que se manifeste l'action protectrice de l'antitoxine présente.

Dans les limites de ces expériences, la mort des animaux ne dépend pas directement du nombre de bactéries présent dans le cerveau, puisque le cerveau des animaux survivant à la suite de l'immunisation contient plus de bactéries que celui des animaux non immunisés qui succombent.

L'interprétation de ces faits doit être faite avec prudence. L'antitoxine n'est jamais pure (6, 19). Elle contient un mélange d'anticorps, avec simplement une proportion plus élevée d'anti-hémolysine *alpha*. Avec les seules données de notre expérience, nous ne pouvons pas affirmer que la protection soit due aux anti-hémolysines exclusivement et non plus à d'autres anticorps.

Par l'étude systématique des phases de l'infection nous pouvons simplement constater que, dans la mort de la souris non immunisée par l'antitoxine, il y a prédominance du mécanisme toxique.

Toutes ces constatations ne sont valables que pour la souris, inoculée par voie intracérébrale. Il est possible que chez l'homme, le mécanisme de l'infection staphylococcique soit plutôt du type bactérien envahissant et moins du type toxique. Le mécanisme initial au moyen duquel les staphylocoques peuvent pénétrer dans les tissus, et surtout s'y établir et s'y multiplier peut être envisagé comme complètement indépendant du mécanisme postérieur et final par lequel ces bactéries, une fois établies en nombre suffisant, causent des dégâts dans les tissus et parfois la mort. On peut émettre l'hypothèse selon laquelle les moments décisifs de l'établissement d'une infection seraient ceux des premiers contacts entre les bactéries et les tissus. Il y aurait autour de la surface des bactéries des substances toutes prêtes à agir qui constitueraient ainsi leur première ligne d'attaque. L'immunité contre ces étincelles qui déclenchent l'"incendie" de l'infection devrait être au moins tout aussi importante que l'immunité contre les produits toxiques libérés en quantité active dans les phases plus tardives où le nombre de bactéries est déjà considérable.

Il est permis d'énoncer pareille hypothèse de travail qui s'est révélée fertile dans l'étude de la gangrène gazeuse à *Clostridium perfringens* (13) et dans d'autres infections (9, 10, 11 et 12).

Quelques auteurs ont déjà envisagé le problème de l'infection staphylococcique sous l'angle bactérien et envahissant, mais les méthodes de l'époque ne les ont pas favorisés (5, 7, 8, 14, et 18). Avec des méthodes comme celle que nous avons employée, il serait possible d'aborder l'étude de l'immunité antitoxique séparément de celle de l'immunité antibactérienne.

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THE OCCURRENCE OF BACTERIA OF THE ARIZONA GROUP IN MAN[1]

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Abstract

The occurrence of bacteria of the Arizona group in man is summarized. Of 87 cultures isolated from man, only 8 were derived from asymptomatic persons whereas 23 were derived from blood and localized infections. Two serotypes, 7:1,2,6 and 10:1,2,5, accounted for more than 50% of the cultures of human origin. Comments are made on the behavior of the organisms in man and the lower animals.

Introduction

The Arizona group as it is now known stems from the isolation by Caldwell and Ryerson (3) of an organism which they called *Salmonella* sp. (Dar-es-Salaam type var. from Arizona) from a disease of Gila monsters (*Heloderma suspectum*) and chuckawalla (*Sauromalus ater*). This bacterium later was designated as *Salmonella arizona* by Kauffmann (9) although he recognized the fact that the organism fermented lactose and liquefied gelatin. The first indications that the Arizona culture typified a definite group of bacteria came from the work of Peluffo, Edwards, and Bruner (11), and of Edwards, Cherry, and Bruner (4).

As described by Edwards, West, and Bruner (6), the Arizona group was made up of cultures of similar biochemical characters which were serologically interrelated. They studied 382 cultures which were divisible into 25 O groups and 61 serologic types. The organisms were distributed widely in reptiles, fowls, and lower mammals, in all of which they seemed to produce definite diseases. The types defined by serology were shown to be epizoologically significant and the organisms were capable of initiating fatal infections in young chicks through feeding and contact experiments. After study of some 1500 cultures, the Arizona group is known to be composed of 32 O groups and approximately 100 serotypes. The organisms resemble salmonellae except that they all liquefy gelatin slowly and practically all ferment lactose. Only one culture has been found which fermented sucrose and only one culture has been observed to produce indol. Occasional cultures produced delayed fermentation of salicin. Numerous serologic relationships to salmonellae involve both the O and H antigens of the group.

Among the 382 cultures studied by Edwards, West, and Bruner (6) only three were isolated from man, but among the 1500 cultures studied to date 87 were of human origin. The occurrence of a number of these organisms in diseases of man was described by the persons who isolated them. However, many were not reported and a summary of the cultures from human sources

[1] *Manuscript received September 26, 1955.*
Contribution from the Communicable Disease Center, Public Health Service, U.S. Department of Health, Education, and Welfare, Atlanta, Georgia.

which were typed in this laboratory has not been published. The purpose of the present paper is to report such cultures and to draw attention to the occurrence of Arizona cultures in infections of man. For methods of serologic characterization and expression of antigenic formulas the reader is referred to Edwards and Ewing (5) and (6).

Observations

The incidence of the various serotypes found in man is given in Table I. The organisms have been found rarely in normal persons. The 1,2:1,2,5 type from an asymptomatic person was isolated from a patient from whom *S. typhi* previously had been isolated. Since no unusual developments were noted during the course of the disease and there was no evidence that the bacterium contributed to the infection, it was recorded as an adventitious organism. The 10:1,3,11 culture was isolated from a 43-year-old midwife concerning whom no exact information was available, as was also true in the

TABLE I

DISTRIBUTION OF ARIZONA TYPES IN MAN

Types	Stools		Blood	Localized infections	Totals
	Asymptomatic	Diarrhea, gastroenteritis, and enteric fever			
1,2:1,2,5	1	7	—	—	8
1,3:1,2,10	—	1	—	—	1
1,4:1,2,5	—	1	—	—	1
1,4:1,2,6	—	1	—	—	1
1,4:1,3,11	—	1	—	—	1
5:1,2,5	—	3	—	—	3
5:1,2,10	—	—	1	—	1
5:13,14	—	1	1	4	6
5:17,20	—	1	—	—	1
7:1,2,6	—	11	5	9	25
9:13,15	—	—	—	1	1
10:1,2,5	—	22	—	—	22
10:1,2,10	—	—	1	—	1
10:1,3,11	1	—	—	—	1
13:1,2,5	—	1	—	—	1
13:13,15	—	1	—	—	1
17:13,15	2	—	—	—	2
17:29-25	—	1	—	—	1
18:13,14	—	2	—	—	2
19:1,2,6	—	1	—	—	1
21:1,2,6	1	1	—	1	3
21:1,2,10	2	—	—	—	2
24:24-28	1	—	—	—	1
Totals	8	56	8	15	87

case of two nurses of the same hospital staff who yielded the 17:13,15 cultures. The 21:1,2,6 type recorded from an asymptomatic person was from the mother of a child said to be affected with salmonellosis. The organism recovered from the child was not identified and it is possible that the mother was a contact of an active Arizona infection. The remaining cultures from asymptomatic persons were isolated from food handlers who gave no history of recent enteric infection.

No attempt was made to separate the organisms from diarrhea and gastroenteritis from those associated with enteric fever-like syndromes. In some instances the separation would have been difficult since persons affected with acute diarrhea were febrile for long periods. In other instances sufficient information upon which to base an opinion was not available. Six of the seven cultures of type 1,2:1,2,5 found in diarrheal cases were representatives of cultures isolated by Verder, Bell, Collins, Schreiber, and Brennan (14) from an outbreak of food infection which affected 51 of a group of 158 student nurses in a Washington, D.C., hospital. Cultures of this type were recovered from 70% of the affected persons but from none of 16 asymptomatic persons examined. Many of the cases were severe and required hospitalization for some days. Rises in agglutinin titer for the Arizona type were observed. The seventh culture of the 1,2:1,2,5 type was isolated from the stool of a 58-year-old woman with severe gastroenteritis. The patient died but no autopsy was performed.

The 1,3:1,2,10 culture was isolated from the stool of a five-month old child with fatal gastroenteritis. The 1,4:1,2,5 culture was isolated from a person affected with diarrhea; the 1,4:1,2,6 culture was found in the stool of an afebrile 25-year-old male who suffered from diarrhea. The 1,4:1,3,11 culture was isolated from the stool of a 3-month-old child with gastroenteritis.

The three 5:1,2,5 cultures were isolated from patients among a group of 32 affected persons. These cultures, received through the courtesy of Dr. S. R. Damon and Mr. A. A. Hajna, were isolated in the investigation of an outbreak of food infection. Thirty-two of 33 people who attended a meal ate cream pie and became ill. The thirty-third person ate cherry pie and was not affected. The three cultures were isolated from three of a small number of the patients who were available for examination. Cultures of the same type were isolated from the pie served at the meal and from the coconut cream mix used in its preparation.

The 5:1,2,10 culture isolated from blood was from an eight-month-old child affected with diarrhea and fever and was described by Seligmann and Saphra (12). The 5:13,14 culture from stools was derived from a 44-year-old man who voided 10 to 15 stools per day for 14 days. The culture of this type from blood, as well as three of the cultures from localized infection, all were isolated from a two-year-old infant. In this child the organisms were known to be present for a prolonged period since the cultures were isolated at intervals covering several months. The organisms localized in the wrist and in the humeroscapular and costochrondral articulations. Marked

destruction of bone was observed. Agglutinins for the isolated organism were demonstrated in the blood. A review of this case was published by Fisher (8). The fourth culture of the 5:13,14 type from localized infection was derived from purulent exudate in otitis media. Again, agglutinins for the organism were present in the blood. An account of the case was published by Butt and Morris (1). The 5:17,20 culture isolated from stools was said to be derived from a person affected with diarrhea. No further history was obtained.

The 7:1,2,6 type was found in 28% of the Arizona infections in man, and in 60% of the cultures from blood and localized infections. Little is known of the individuals from whose stools this type was isolated except that they were said to be affected with diarrhea. Six were derived from adults and one of these was isolated from a 33-year-old male who voided 30 to 40 stools per day. The patient was febrile and pus and blood were present in the stools. No histories of the remaining five patients were obtained. Of the 7:1,2,6 cultures from blood, two were isolated from an adult female at intervals of four months. The patient ran a spiking temperature and the organism was isolated from the blood on several occasions. The duration of the infection was estimated at eight months. Lupus erythematosus also was present in this individual. The remaining cultures of 7:1,2,6 from blood were isolated from infections in adults but no other information could be obtained.

Of the 7:1,2,6 cultures from localized infections, one was from a brain abscess in an adult who had been ill for four years with intermittent diarrhea, headache, and low grade fever. The patient also had been affected intermittently with thrombophlebitis. The final outcome of the case was not learned. A second culture of this type was isolated from a liver abscess at autopsy. One culture was from pleural fluid of a 72-year-old male affected with pleurisy, cyanosis, and dyspnea. Two cultures were isolated from the urine of adult males affected with severe cystitis and prostatitis. In one of these persons purulent pyelitis also was observed. Of the remaining four cultures, one each was isolated from a scalp abscess, an abscess on the right thigh, spinal fluid, and exudate from severe conjunctivitis. The latter infections occurred in four adult individuals.

No information was available concerning the 9:13,15 culture except that it was isolated from an hepatic abscess at autopsy.

Of the 22 cultures of the 10:1,2,5 type isolated from diarrhea, 10 were isolated from two outbreaks of infection described by Murphy and Morris (10). The first occurred among eight children of three families who had eaten a common lunch. Six of the children became acutely ill and were hospitalized. The Arizona type was isolated from all of the affected children. Ice cream was thought to be the vehicle of infection. Agglutinins for the organism were demonstrated in the blood of these children. The second outbreak of 10:1,2,5 infection occurred among six patients in a small hospital ward for whom a special beverage was prepared by an attendant who was just recovering from

an attack of diarrhea of three days' duration. The organism was recovered from three of the six patients who were affected with fever, vomiting, diarrhea, and prostration and from the stool of the attendant who prepared the food.

Of the remaining 12 cultures of the 10:1,2,5 type, seven were from infants acutely ill with diarrhea and enteritis. Two of the infants were siblings affected at the same time. From one of the five sporadic cases in infants, *Shigella flexneri* 2 also was isolated. This is the only instance in the series of human infections here recorded that a known pathogenic form was found in association with any of the Arizona serotypes. Five of the 10:1,2,5 cultures were from four sporadic cases in adults, all of whom were affected with fever, diarrhea, and vomiting. Two of the cultures were isolated at an interval of three days from a 36-year-old farmer who was hospitalized. One of the adults yielding type 10:1,2,5 had eaten ice cream from which the organism also was isolated.

The 10:1,2,10 type from blood was isolated from the heart blood of a five-month-old infant at autopsy. The 13:1,2,5 and 13:13,15 types were isolated from children with diarrhea, the first in the Philippine Islands and the second in Brazil. The only information obtained concerning the 17:29-25 type was that it was isolated from a patient with diarrhea. The 18:13,14 cultures were isolated from two widely separated sporadic cases in adults. Both were affected with fever and vomiting as well as with diarrhea. The 19:1,2,6 type was isolated from the stool of an infant hospitalized for two weeks and affected with diarrhea, fever, and dehydration. The 21:1,2,6 culture from diarrhea was isolated by Dr. W. Hirsch in Israel and was forwarded to the writers by Dr. F. Kauffmann. The culture from localized infection, also received from Dr. Kauffmann, was isolated from pleural fluid of a 42-year-old male affected with pleurisy.

Comments

The fact that a given group of bacteria is found in the stools of persons affected with diarrheal disease obviously is not valid evidence that they cause enteric infections. However, when a bacterial group has been found to produce enteric infection very frequently in lower animals, when the bacteria commonly invade the blood and produce fatal infections with high mortality rates in young animals, when serotypes of the group defined by antigenic characterization have been demonstrated to be epizoologically significant, and when it has been demonstrated by feeding and contact experiments that the organisms produce transmissible enteric infections in animals, their presence in persons with enteric infections must be regarded with extreme suspicion. Further, when the organisms occur in the absence of other recognized pathogenic forms, are found very infrequently in asymptomatic persons, and have a marked tendency to invade the blood and to produce localized infections, little doubt remains that they are etiologically significant. Their presence in mass food infections and the production of agglutinins in affected persons furnish ancillary evidence of their relationship to human infections.

In addition to their occurrence reported in the publications cited in the previous section, Arizona cultures have been found in human infections by Ferris, Hertzberg, and Atkinson (7) who isolated identical cultures from 26 of 29 persons affected with fever, diarrhea, and vomiting. Although the organisms were not identified as to type, the biochemical and serological properties of the cultures were characteristic of Arizona strains. Buttiaux and Kesteloot (2) recognized Arizona types in six sporadic cases of enteric infection, some of which exhibited symptoms of gastroenteritis and others a typhoidal syndrome.

The cases of Arizona infection which have been recognized seem to be just as severe, if not somewhat more severe, than the usual case of salmonellosis. In the present series, 26% of the cultures were isolated from blood or localized infections, a rate much higher than is observed in infections with those *Salmonella* serotypes which typically produce diarrhea and gastroenteritis in man. Only those types of *Salmonella* particularly adapted to man (e.g. *S. typhi*, *S. paratyphi* A, etc.) and *S. cholerae suis* are found so frequently in the blood and in localized infections. In particular, type 7:1,2,6 seems to possess a marked tendency to invasion since 56% of the cultures were isolated from extraintestinal sources. Such observations raise the question as to whether Arizona types actually are more invasive than the majority of *Salmonella* serotypes or whether a large percentage of lactose fermenting bacteria from stools are discarded whereas those from other sources are subjected to more careful scrutiny and are more likely to be submitted for exact identification. In this connection it is perhaps significant that the cultures of type 10:1,2,5 described here, all of which were isolated from stools, did not ferment lactose.

Practically all of the cultures described here fermented lactose quite slowly. It was not until Solowey and McFarlane (13) systematically studied all cultures isolated from egg powder that it was realized that Arizona cultures may produce acid and gas from lactose within 24 hr. This situation can but lead to speculation as to the number of Arizona cultures which are never picked from fecal plates or which are discarded when placed on differential media. This situation is comparable to the realization that certain *E. coli* serotypes are associated with infantile diarrhea and emphasizes the pitfalls inherent in adherence to lactose fermentation as an absolute screen in enteric bacteriology.

It seems apropos to comment on the occurrence in animals of the two most frequently occurring types in this series, 7:1,2,6 and 10:1,2,5. At the time of the last published summary of the occurrence of Arizona serotypes (6) only two cultures of type 7:1,2,6 had been found. Therefore, it was very surprising when this type began to be found repeatedly in man. The first cultures of human origin were isolated in California and were received through the courtesy of Dr. A. S. Browne. No systematic typing of Arizona cultures from animals in California had been done for several years. When examination of animal strains from this area again was initiated, it was found that the

7:1,2,6 type had become widely distributed in fowls in that state and to date 162 cultures of type 7:1,2,6 have been recognized. This furnishes some basis of explanation for its repeated occurrence in man in that area. The serotype no longer is confined to the west coast but has been found repeatedly in birds in the midwest; more recently it was recognized among cultures from fowls in eastern states, where an infection in man also occurred. One can but contrast the occurrence of type 7:1,2,6 with another type of the same O group, 7:1,7,8. The latter serotype originally was found in but one hatchery in California. Its spread through sale of hatching eggs was traced to the midwest and to the eastern seaboard. It has become firmly established in fowls throughout the United States and 582 cultures of this type have been found in animals. Yet this type has never been found in man. Such observations lead one to speculate whether host adaptation of serotypes occurs in the Arizona group as among salmonellae.

The 10:1,2,5 type was represented by only four cultures in 1947 whereas 129 now have been recognized. This type was prevalent in a very large breeding flock of hens in North Carolina. Eggs from this flock were used to establish supply flocks for hatcheries in Indiana and Georgia which sold large numbers of chicks in the southeastern states. Soon this serotype began to appear in chicks, cats, and dogs in that area and it was soon thereafter that infections in man occurred. The majority of cultures of this type from man occurred in the southeastern part of the country. Thus, the available evidence indicates that animal reservoirs have some bearing on the appearance of the bacteria in infections of man.

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THE IMPORTANCE OF URINARY ANTIBODIES IN THE DIAGNOSIS OF LEPTOSPIROSIS[1]

By R. D. Stuart

Abstract

Low-titer antibodies have been found with great regularity in the urine of patients suffering from Weil's disease (*Leptospirosis icterohaemorrhagiae*). These antibodies were present in a majority of patients by the 10th day of illness and were almost invariably found in the second and third weeks of disease. Removal of antibodies from the urine by absorption on heat-killed non-virulent *L. icterohaemorrhagiae* significantly increased the number of successful isolations by guinea pig inoculation. Non-specific adsorption of the urinary protein by kaolin was also effective in reducing the antibody titer and this is suggested as a more generally applicable technique.

The urinary antibodies were often detected in the absence of a clinically demonstrable proteinuria. This was found to be due to the relatively larger proportion of globulin excreted by the kidney. In the absence of any gross proteinuria, antibodies to the infecting leptospiral serotype were dominant in the urine, both in human infections with *L. icterohaemorrhagiae* and in canine infections with *L. canicola*. Thus the remarkable specificity of the urinary antibodies was very helpful in determining the type of infecting leptospira when ordinary serological tests gave confusing cross agglutinations. Speculation on the phenomenon led to the suggestion that it might be due rather to a qualitative difference between specific and non-specific antibodies than to local production of specific antibodies in the kidney itself.

Introduction

Except in regions where Weil's disease is prevalent, clinical suspicion of leptospirosis is rarely aroused on clinical grounds before the end of the first week of illness. At this stage the laboratory diagnosis depends on the demonstration of a rising titer of specific serum antibodies, preferably supplemented by isolation of the organism from the patient. Both procedures may appear simple, as indeed they are in individual instances, but on occasion both can be extremely difficult. The serological tests may be complicated by considerable cross agglutination or even by paradoxical agglutinations (4, 17), in which titers to heterologous strains are higher than to homologous. The isolation of leptospirae from blood is rarely successful after the first week of illness and their isolation from urine, in which they appear with considerable regularity, is not so easy as commonly believed. Animal inoculation is the method of choice, but the success of the procedure may be prevented by factors, such as urinary acidity, which interfere with the viability of the organisms, and the natural resistance of the test animal, which in turn depends on age and species. The main factor, however, is the age of the specimen itself. So important is this that authorities such as Wolff (19) still suggest that animals should be inoculated at the patient's bedside and Yager and Gochenour (20) have been so impressed by the results obtained in this way

[1]*Manuscript received September 30, 1955.*
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that they have abandoned their excellent technique of high speed centrifugalization to follow this method which dates back to the earliest days of leptospiral investigation.

Observations on the excretion of antibodies in the urine are pertinent to both problems, and an appreciation of their significance can be of considerable help in diagnosis, particularly in *L. icterohaemorrhagiae* infections.

The Occurrence of Urinary Antibodies in Weil's Disease

Antibodies have often been demonstrated in the urine of patients with leptospirosis. Inada, Ido, Hoki, Kaneko, and Ito (6), in one of the earliest papers on *L. icterohaemorrhagiae* infection, pointed out that specific antibodies were excreted in the urine during the course of the disease. Van der Hoeden (14, 15), who is much quoted in this context, found antibodies in the urine of 26 out of 27 human cases or convalescents and in 40 out of 45 patients who had recovered. Antibodies appeared usually two or three days after they were demonstrable in the blood and occasionally could be detected two or three weeks after recovery. The titers varied from 1:4 to 1:256 in patients and convalescents and from 1:2 to 1:128 in recovered cases. In 100 observations on 52 cases, Davidson and Smith (2) found "in the first week of the illness 3 out of 18 (16.6%), in the second week 20 out of 26 (71.4%), in the third week 34 out of 37 (91.8%), and after the third week 17 out of 17 (100%) gave positive reactions in dilutions of 1:2 or greater." The highest titer found was 1:300. Borgen (1*a*) found specific antibodies in 25 out of 26 samples of urine from eight patients. All the patients excreted antibody at some period of their illness and the titers were on the average about one per cent of the corresponding serum titers. Similar findings were recorded by the author on cases investigated in Glasgow, Scotland, between 1938 and 1944 (Table I).

Fifty-eight specimens from 29 patients were examined. Most of the examinations were sporadic because the possible significance of these antibodies was not obvious over most of the period. The recorded titers, however, are in full accordance with the findings of the workers quoted above and show very clearly the increasing incidence and rise of titer with increasing duration of illness.

TABLE I

OCCURRENCE AND TITERS OF URINARY ANTIBODIES IN WEIL'S DISEASE

Day of illness	10–15	16–20	21–25
Negative	4	1	0
Positive	18	19	16
Titer 1:4	3	1	0
1:8	8	1	1
1:16	4	6	1
1:32	2	3	5
1:64	1	6	4
1:128	0	2	3
1:256	0	0	2

The Isolation of *L. icterohaemorrhagiae* from the Patient's Urine

Before serological methods reached their present efficiency, the demonstration of a leptospiruria was the most important diagnostic procedure in Weil's disease. Uhlenhuth and Fromme (13) found leptospirae in the urine as early as the third day of illness and Ido, Hoki, Ito, and Wani (5) as late as the 63rd day. Most authors found that microscopic examination of urinary sediment was much more successful than animal inoculation. Inada *et al.* (6), for example, were able to infect guinea pigs in only one third of a series of microscopically positive specimens. Since the final proof of the nature of the illness depends on the isolation and identification of the infecting leptospira, much effort was put into determining the cause of this failure. Early workers such as Noguchi (9) stressed the harmful effect of acidity. This can be avoided by neutralizing the urine or better by giving patients a simple alkaline mixture for 24 to 48 hr. prior to the examination. The influence of bile, also stressed by early workers, could not be confirmed. Natural resistance of the inoculated animals, however, was found to be quite important, young animals being more susceptible (3, 18). Guinea pigs under 200 gm. are highly susceptible to *L. icterohaemorrhagiae*, though young hamsters are usually preferred if other spirochaetes are likely to be encountered. Van der Hoeden (16) has suggested that certain species of gerbils may be even more successful in use. In spite of attention to all these details of technique, Davidson and Smith (2) who examined 211 specimens of urine from 88 cases of Weil's disease were able to infect guinea pigs with 19.3% of samples taken between the 8th and 14th day of illness, 21.5% between the 15th and 21st day of illness, and only 15% between the 22nd and 28th day. These workers laid considerable stress on the probable influence of antibodies on their findings and showed experimentally that the addition to normal urine of antiserum to a titer of 1:30 killed leptospirae very rapidly.

The Effect of Urinary Antibodies

This could readily be studied by the inoculation of artificially prepared specimens with and without antibodies. The investigation of natural specimens presented more difficulties. Dilution of the urine with physiological saline, which is often recommended as a practical expedient to lower antibody concentration, was not quite satisfactory, but it seemed feasible to prepare comparable parallel specimens by specifically absorbing the antibodies from one of them. Preliminary experiments indicated that heat-killed *L. icterohaemorrhagiae* cultures specifically absorbed antibodies from patients' urine and that leptospirae would remain alive in such urines whereas they died rapidly in the same urine containing antibody. The technique was then applied to the isolation of leptospirae from a series of patients with Weil's disease.

Urine of neutral or alkaline reaction was obtained directly from patients and was centrifuged for 20 min. at 3000 r.p.m. The deposit and a small volume of the immediately adjacent layer of the supernatant (which often

TABLE II

THE EFFECT OF SPECIFIC ABSORPTION OF URINARY ANTIBODIES

Day of illness	Titer of urinary antibodies*		Fatal leptospiral infection in inoculated guinea pig	
	Ordinary	Absorbed	Ordinary	Absorbed
13	16	0	0	+
13	16	4	+	+
15	32	8	0	+
16	4	0	+	+
17	64	8	0	+
18	16	0	+	+
18	0	0	+	+
19	64	16	0	+
19	32	4	0	+
22	256	64	0	0
23	256	128	0	0
29	64	16	0	0
30	8	0	0	+

**Expressed as reciprocal of dilution.*

contains the more actively motile spirochaetes) were then pipetted off and divided into two equal portions. One was inoculated immediately, generally in 2 ml. volume, into the peritoneal cavity of a young guinea pig. To the other was added an equal volume of a rich culture of *L. icterohaemorrhagiae*, the non-virulent strain Wijnberg, previously heated to 60° C. for one hour. This mixture was allowed to stand for a few minutes and then a volume of 2 ml. of it was inoculated into a similar animal. Table II shows the results obtained.

In no instance were leptospirae isolated from ordinary or absorbed urine which had a final antibody titer greater than 1:16, but absorbed urine produced infection in 10 cases out of 13, whereas ordinary urines from the same patients were successful only in four cases. While it is true that the majority of the guinea pigs inoculated in the usual way received twice as much urine as those inoculated with the absorbed specimen, this certainly does not invalidate the comparative value of the results. The difference must be due entirely to the removal or reduction of antibody in the absorbed specimens.

The effect of this absorption on leptospiral viability was also demonstrated in the isolation of the spirochaete from rats' kidneys. The addition of even tiny portions of cortex to ordinary leptospiral culture media led to a gradually increasing titer of antibodies in the fluid. This could be entirely prevented by the simultaneous introduction of a small quantity of killed non-virulent leptospirae. From seven rats examined by this method leptospirae were recovered in four instances, twice as many as by parallel routine culture. The cultured leptospirae were all virulent.

TABLE III

A COMPARISON OF TWO METHODS OF REMOVING URINARY ANTIBODIES

Case	Day of illness	Urinary antibody titers: Original	After culture absorption	After kaolin adsorption	Amount of kaolin used
T.K.	16*	16	—	0	0.5 gm. in 18 cc.
	20*	16	0	0	0.5 gm. in 20 cc.
	21*	32	0	4	0.5 gm. in 20 cc.
J.A.	35	512	—	128	0.5 gm. in 20 cc.
	56	64	0	8	0.5 gm. in 20 cc.
McN.	19†	0	0	0	0.5 gm. in 25 cc.
	48	64	—	64	1.0 gm. in 20 cc.
	57	32	0	0	1.0 gm. in 20 cc.
McK.	14†	8	—	0	1.0 gm. in 25 cc.

**Inoculated guinea pig died from an intercurrent infection.*
†Guinea pig inoculated with each sample died from Weil's disease.

The application of this absorption technique in clinical practice is obviously difficult because it can be carried out only in laboratories where leptospiral cultures are regularly available. However, since leptospiral antibodies are presumably associated with serum proteins excreted in the urine, it seemed possible that removal of protein by a non-specific adsorbent might be reasonably effective and more readily applicable. Experiments were accordingly carried out by adding charcoal or kaolin (15) to urines of low antibody titer produced by the addition of immune serum. Both substances reduced these titers to some extent, kaolin being the more effective. This substance did not interfere with the viability of leptospirae in artificially inoculated urines. Four human cases of Weil's disease became available for direct experiment. The effect of kaolin adsorption and of culture absorption in a number of instances is shown in Table III.

The result, however, was quite inconclusive. Two sets of animals died from typical *L. icterohaemorrhagiae* infection, in each instance the ordinary and absorbed urines had low or negligible titers. Three sets of inoculated animals died from a coincidental *S. typhi-murium* epizootic. The others remained healthy. All this investigation demonstrated was that kaolin had no obvious detrimental effect on the virulence of leptospirae. No further opportunity occurred to investigate the technique, though more information on its value, particularly in relation to the transport of urines which cannot be brought to the laboratory immediately, seems especially desirable.

The Relationship of Urinary Antibodies to Urinary Protein

Many workers have pointed out the correlation that exists between the presence of antibodies in the urine and the almost invariable presence of albuminuria, and the hypothesis that there is a causal relationship seems

TABLE IV

THE LACK OF RELATIONSHIP BETWEEN URINARY PROTEIN AND ANTIBODY TITER

Case: Weil's disease, male 38 years

Day of illness	Urine			Blood
	pH	Protein	Antibody titer	Serum titer
12	8.2	Tr.	1/8	1/10,000
15	7.8	0	1/32	
17	7.5	0	1/64	
19	7.0	Tr.	1/64	1/10,000
25	6.0	0	1/16	
27	6.0	0	1/32	1/10,000

eminently reasonable. However, antibodies may be present in quite high titer without any demonstrable albuminuria. Such a finding was recorded by Borgen (1*b*) and an example from our own records is summarized in Table IV.

Urine was acidified if necessary, filtered, and tested with sulphosalicylic acid. Complete absence of even the slightest trace of precipitation was recorded as negative. Certainly this does not rule out the presence of small amounts of protein, as was shown by the simple device of evaporating one of the above negative urines (17th day) at room temperature. By this means 25 ml. of urine was reduced in volume to 5 ml. A faint precipitate with sulphosalicylic acid was then obtained. This was decidedly less than that obtained in 1:1000 dilution of blood serum from the above case. Thus the urinary proteins, if derived directly from the blood, represented a dilution greater than 1:1000 and probably close to 1:5000. Urinary antibody, however, represented a serum antibody dilution of only 1:150. Obviously the antibody/protein relationship in the urine was not the same as in the serum and it seemed probable that urinary protein was qualitatively different from serum protein. This was borne out by the investigation of another case, unfortunately not quite so satisfactory in that demonstrable protein was present in the urine. In this patient the urinary protein estimated by sulphosalicylic acid was less than 1:1000 of the serum protein, while the urinary antibody was between 1:100 and 1:200 of the serum antibody. This investigation was carried out independently by the hospital biochemist, Dr. A. Bruce Anderson. All protein estimations were by the biuret method. In the final dialyzate the proportions of albumin to globulin were 44 to 126, approximately 1:3. A later urine from the same patient contained more protein and the discrepancy between the protein and antibody content was less marked. In this specimen the albumin–globulin ratio was 1 to 0.74. In both specimens the protein excreted contained a relatively greater amount of globulin compared to blood serum. Since antibodies are particularly associated with the globulin fraction, this finding explained the peculiar antibody/protein ratio. What significance it has on the next phenomenon remains to be determined.

The Specificity of Urinary Antibodies

The specificity of urinary antibodies in relation to leptospiral disease is unquestionable. Such antibodies have never been demonstrated in persons or animals without some other evidence of leptospiral infection and in the absence of concurrent serum antibodies. In cases with a frank proteinuria, the antibodies present in the urine, with appropriate allowance for dilution, parallel the antibodies in the serum very closely. When urinary protein is absent or small in amount relative to the urinary antibody titer, a narrower specificity may become evident. That is, when the discrepancy referred to above in the antibody/protein ratio becomes evident, and when the presumed qualitative difference in the urinary proteins is present, a qualitative difference is often apparent between the antibodies in the urine and in the serum. Table V makes this clear.

All the human cases were proved to be *L. icterohaemorrhagiae* infections, and all the canine were *L. canicola* infections. The fifth human case simply demonstrates the common relationship of cross agglutination in serum and urine in the presence of a frank proteinuria. The other specimens, human and animal, came from cases in which the relative serum titers to specific and non-specific organisms were sufficiently close to make the diagnosis questionable, or, when the titers were identical, to render direct serological diagnosis impossible. Absorption tests would undoubtedly clarify the situation, but these are clumsy and impracticable except in a specialized laboratory. The diagnostic value of the greater specificity of urinary antibodies is unquestionable. Cross agglutination to equivalent serum titers with specific and non-specific organisms, particularly *L. icterohaemorrhagiae* and *L. canicola*, is common in both human and canine disease. Paradoxical agglutination with the higher serum titer to the non-specific strain is occasionally encountered,

TABLE V

COMPARISON OF SERUM AND URINARY ANTIBODIES

Case	Serum titer		Urinary titer		Protein (sulphosalicylic acid)
	"ictero"	*canicola*	*"ictero"*	*canicola*	
			Human		
1	30,000	10,000	64	0	0
2	30,000	10,000	64	0	Ft. tr.
3	10,000	10,000	64	0	0
4	3,000	3,000	16	0	0
4	3,000	3,000	8	0	0
5	30,000	3,000	128	32	+
			Canine		
1	10,000	60,000	8	128	+−
2	3,000	10,000	4	128	+−
3	3,000	3,000	8	64	Tr.
4	3,000	3,000	16	32	+−

particularly in the early stages of illness. Neither phenomenon has been encountered in the titration of urinary antibodies. The number of observations, however, has been limited because the phenomenon was observed late in this investigation and cases of this kind are no longer available for study.

Discussion

Murray (7) makes a plea for constructive speculation in scientific literature and so, in a journal inscribed to his honor, one may be allowed to venture on speculation in the hope that some of it may eventually deserve the qualification of "constructive".

The first speculation centers on the effect of antibodies on leptospirae within the urinary bladder. The fact that removal of antibody from the urine increases the chances of a successful isolation is evidence that the antibody has an inhibitory effect on these organisms; yet in the bladder leptospirae must often remain viable even in the presence of fairly high titers of antibodies over a number of hours. It should be remembered that *in vitro* these antibodies may produce either agglutination or lysis of the organisms, and that the latter effect takes place entirely in the absence of complement. The agglutinative effect, as is well known, is not necessarily lethal but the lytic action certainly is. The observations of Borgen (1*c*) may be pertinent to this problem. He points out that the effect of antibody in fresh urine is demonstrable more as an agglutination than as a lysis and therefore is not accompanied by death of the spirochaete. This can be demonstrated best by using normal urine as the diluent in serological tests. Observations on the influence of the suspending fluid on the lytic action of leptospiral antibodies have also been reported by the author (12), who noted the elimination or inhibition of lysis by certain concentrations of electrolytes or hydrophilic colloids. The results suggest that the lytic phenomenon is a physical as much as an immune reaction. Thus the destruction of leptospirae within the bladder may well be inhibited by physical factors, at least for a considerable period.

The inhibition of lysis and thus of leptospiral destruction, if it occurs in the bladder, should continue to exercise an influence in the voided urine, so it is difficult to explain the necessity, derived from the experience of so many workers, of injecting urine into animals immediately it is passed by the patient. A delay of a few hours in injecting the urine would not affect its antibody content, so passive immunization does not explain the observed difference. It may well be that the manipulations usually associated with the inoculation of urine after it has been allowed to stand, particularly centrifugalization and dilution of the deposit with saline, may accelerate lysis of leptospirae already affected by antibody. Nevertheless, the success of the specific absorption experiments lends strong support to the belief that the continuing effects of antibody in excreted urine are detrimental to leptospirae. The absorption of antibodies should extend the viable period of leptospirae and thus prolong the time within which effective isolation can be practiced. This technique may prove of value in the practical problem of isolating the organisms from the urine.

Furthermore, the use of kaolin or other non-specific absorbent may overcome the present difficulty of transporting the specimen to a far distant laboratory.

The diagnostic value of urinary antibodies is considerable because of their remarkable specificity. For ordinary purposes the demonstration of serum antibodies is simpler, because these show higher titers and occur earlier in disease; but when equivalent serum titers occur to more than one strain or when possible paradoxical reactions are encountered, a simple serological test on the urine may resolve the difficulty. Difficult cases of this type were encountered, including both *L. icterohaemorrhagiae* infection in man and dogs and *L. canicola* infection in dogs, and in these cases urinary antibody determination usually provided a reliable method of differentiation. In a few instances it was unsuccessful because of the absence of urinary antibodies or because the disturbance of kidney function was so gross that the urinary antibodies simply expressed a direct leakage of serum protein into the urinary tract.

The suggestion that the high relative concentration of antibodies in the urine is due to a qualitative difference between urinary and serum proteins is supported by modern physiological concepts. Rigas and Heller (11) show that in man there is normally a constant proteinuria and that the albumin–globulin ratio of the urinary protein is the reverse of that in the plasma. Oliver, MacDowell, and Lee (10) demonstrate the absorption of albumin by the renal tubules of rats and incidentally point out that the urinary protein is derived from the plasma protein presumably by passage through the glomerular membrane since it is impossible to produce proteinuria in animals that have no glomeruli. The urinary globulin is thus derived from the serum globulin and the urinary antibodies should be the antibodies normally associated with these globulins. This does not explain the narrow specificity of these antibodies in leptospirosis. There seem to be two possibilities: either that they are produced locally within the urinary tract, or that they are selectively filtered through the kidney. The first theory is supported by the observations of Naylor and Caldwell (8) on the remarkable specificity of urinary antibodies in typhoid carriers even when antibodies to other salmonellae are present to high titer in their sera. However, even if this theory is true, selective filtration of antibody must also be accepted as a possibility because serum globulins are undoubtedly passing through the kidney but only those carrying specific antibody configuration are being excreted. In leptospirosis it is not necessary to postulate local production of antibody because there seems to be a qualitative difference between specific antibody produced to the infecting strain and para-specific antibody. The latter may suggest infection with another leptospiral serotype because it is often completely disproportionate to the amount of antigen common to the two strains. In such cases it is easy to absorb para-specific antibody by the specific organism. However, it is never easy to absorb specific antibody by the organism identified with the para-specific antibody, even if the titers of these antibodies are identical. Gispen and Schuffner (4) also argue from the difference in the time of appearance and disappearance of these antibodies

that qualitatively different processes are concerned with their production; and in this they are supported by van Thiel (17). This leaves us with a fascinating speculation about the constitutional differences between specific and non-specific antibody molecules, a difference which can be distinguished by the human kidney. Electrophoretic investigation should throw new light on this subject.

Acknowledgment

Most of these investigations were carried out in the Bacteriology Department of the Royal Infirmary, Glasgow, Scotland, and I wish to express my appreciation of the facilities provided. The helpfulness of the hospital biochemist has been indicated in the paper but the value to the investigation of the specimens contributed by my friends from all over the southwest of Scotland has not been previously acknowledged.

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THE DEVELOPMENT OF INCLUSIONS IN TISSUE CULTURES OF MONKEY KIDNEY EPITHELIAL CELLS INFECTED WITH POLIOMYELITIS VIRUS[1]

By A. J. Beale, Patricia F. Stevens, Norma Davis, W. Stackiw, and A. J. Rhodes

Abstract

A cytoplasmic inclusion body has been found in the epithelial cells of monkey kidney grown in tissue culture and infected with poliomyelitis virus. This inclusion is at first closely applied to the nucleus, and later develops into a clearly demarcated structure. The nucleus is pushed to the periphery of the cell and becomes pyknotic. Finally, the cytoplasm around the inclusion becomes vacuolated, and the cell breaks up at about the time virus first appears in the fluid part of the infected tissue cultures. Multiple small intranuclear eosinophilic inclusion bodies have also been found in some cells that contain cytoplasmic inclusions.

Introduction

Barski *et al.* (2) have described a large cytoplasmic inclusion body in the fibroblast cells that grow in tissue cultures of human tonsils infected with poliomyelitis virus. An apparently similar type of inclusion has been reported by one of us in tissue cultures of monkey kidney epithelium (15). This cytoplasmic inclusion has been found regularly in cultures of monkey kidney cells infected with all three types of poliomyelitis virus which have been examined in the past year. Strains freshly isolated from patients, as well as stock laboratory strains, produce the inclusion.

Cultures of monkey kidney tissue subjected to trypsin extraction and grown on cover slips are well suited to the investigation of intracellular changes during the growth of virus. Such cultures can be infected with a large dose of virus, washed to remove unabsorbed virus, and fixed and stained after varying periods of incubation. Parallel determination of the amount of virus in the extracellular fluid of the cultures may be made by titrations in tissue cultures.

The present paper reports the results of such an investigation, and furnishes a more detailed description of the inclusions already referred to earlier (15).

Materials and Methods

Preparation of Tissue Cultures

Trypsinized monkey kidney cultures were prepared by Melnick's modification (13) of the methods described by Dulbecco and Vogt (7) and Youngner (17). In most experiments, growth of epithelial cells was initiated in synthetic Medium 199 (14) containing 2% horse serum; in later experiments, 0.5%

[1]*Manuscript received October 1, 1955.*

Contribution from The Department of Paediatrics, University of Toronto, and The Research Institute, The Hospital for Sick Children, Toronto, Ontario. Assisted with funds allocated by the Province of Ontario under the National Health Grant Program of the Department of National Health and Welfare, Ottawa.

PLATE I

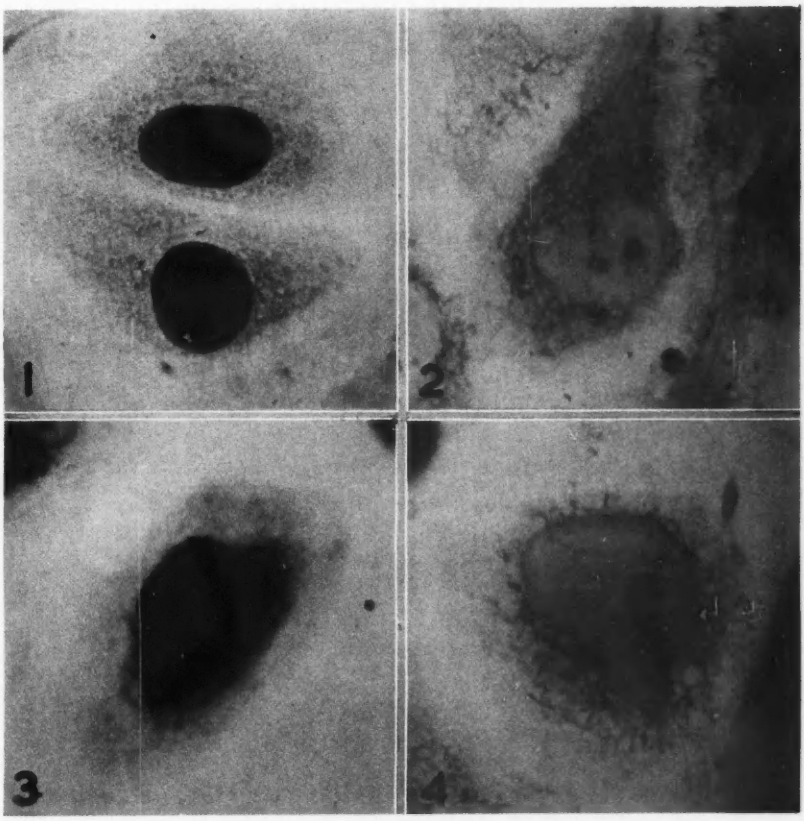

FIG. 1. Normal monkey kidney cells (H & E). (× 1100)

FIG. 2. Normal monkey kidney cell showing mitochondria (Bensley–Cowdry method). (× 1100)

FIG. 3. Monkey kidney cell showing initial stage of development of inclusion body four hours after infection with poliomyelitis virus (Giemsa). (× 1100)

FIG. 4. Monkey kidney cell showing the polar accumulation of mitochondria in a cell infected four hours previously with poliomyelitis virus (Bensley–Cowdry method). (× 1100)

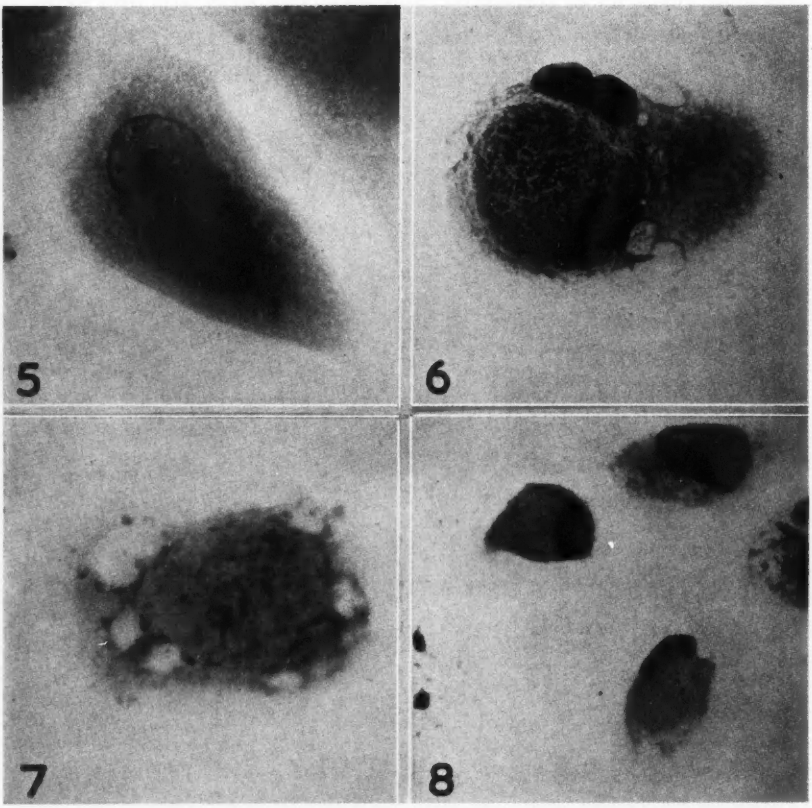

FIG. 5. Monkey kidney cell showing well developed cytoplasmic inclusion, also three intranuclear inclusions (H & E). (× 1100)

FIG. 6. Large cell showing well marked inclusion with fat globules. The nucleus is pyknotic and the cytoplasm beginning to be vacuolated: six hours postinfection (osmium tetroxide, Giemsa). (× 750)

FIG. 7. Monkey kidney cell showing vacuolation of the cytoplasm and thickened mitochondria in the inclusion and around the vacuoles (Bensley–Cowdry method). (× 1100)

FIG. 8. Final stage showing remnants of cytoplasm and pyknotic nuclei (H & E). (× 1700)

lactalbumen in Hanks' balanced salt solution, with 2% horse serum, was used. Penicillin (500 units/ml.) and streptomycin (250 micrograms/ml.) were added to all media.

Virus

The Mahoney strain of Type 1 virus was used in the experiments in which the details of development of the inclusions were studied; the virus was in the form of a "pool" of tissue culture fluid stored at −20°C. In addition, strains MEF 1 (Type 2) and Saukett (Type 3) were studied only with regard to the morphology of the inclusions, as were several freshly isolated strains of Types 1 and 3.

Virus Titrations

Serial 10-fold dilutions of Mahoney virus were made in Medium 199 and 0.1 ml. aliquots were added to tubes containing well-grown cultures of kidney epithelium. Five tubes were inoculated with each dilution, and the results were read after one week. The dilution of virus causing specific degeneration of 50% of the cultures was calculated by the Kärber method, and expressed as the 50% cytopathogenic dose (CPD_{50}) per ml.

Fixation and Staining

Cells were grown on cover slips 11 × 40 mm. so that cultures could be conveniently fixed and stained. Fixation was usually carried out in a mixture of methyl alcohol 85%, formalin 10%, and glacial acetic acid 5%, and cover slips were then stained with Harris's haematoxylin and counterstained with eosin. Some cover slip cultures were fixed in osmium tetroxide vapor for about 10 minutes, stained with Giemsa, and differentiated with acetone-xylol.

Examination for Mitochondria

Cover slip cultures were fixed in Regaud's fluid for 24 hr. and then treated with potassium bichromate for eight days. After thorough washing in distilled water, they were stained with the Bensley–Cowdry acid fuchsin methyl green stain or with iron haematoxylin (4).

Study of Inclusions

Cover slip cultures with a good growth of epithelial cells were infected with 2 ml. of Mahoney virus pool, containing about 10^7 CPD_{50} per ml. Half to one hour later, the fluid was removed from the cultures, and the cells were washed three times with 2-ml. quantities of Medium 199. The fluid was finally replaced with fresh Medium 199. Two or more cultures were removed at hourly intervals thereafter; the fluids were pooled and titrated for infective virus, and the cover slips were fixed and stained.

The normal appearances of monkey kidney monolayer cultures stained by haematoxylin and eosin and for mitochondria are shown in Figs. 1 and 2. The epithelial cells contain a spherical nucleus with one or two nucleoli and

uniformly distributed chromatin. The cytoplasm is finely granular and contains small lipid vacuoles. Filamentous mitochondria are distributed throughout the cytoplasm.

In Mahoney virus infected cells, the initial change appears to be a condensation of the cytoplasm around one pole of the nucleus, between three and four hours after infection (Fig. 3). Sometimes it is difficult to distinguish the developing inclusion from the nucleus to which it is closely applied. The mitochondria appear shortened and are concentrated around the nucleus (Fig. 4). Later, the inclusion appears as a purple structure which sometimes indents the nucleus, and is clearly demarcated from the surrounding cytoplasm (Fig. 5). In some cells, as shown in Fig. 5, two or three small eosinophilic inclusion bodies have been found in the nucleus. These have only been seen in a few cells also containing cytoplasmic inclusions.

The infected cells increase in size as the inclusion develops, and the nucleus is pushed to the edge of the cell; later, the cytoplasm at the periphery of the cell becomes vacuolated. The inclusion body contains many lipid granules staining black with osmic acid (Fig. 6). Mitochondria are present in the inclusion, but appear to be degenerating in the later stages (Fig. 7). Finally, the cell disintegrates as the vacuolation of the cytoplasm increases, leaving the remains of the cytoplasm and pyknotic nucleus (Fig. 8).

It was relatively easy to follow the sequence of infection. However, in the later stages of infection, several forms of inclusion body were represented in one cover slip culture.

The appearance of the inclusion body always preceded the liberation of virus into the culture fluid. Table I shows the results of three experiments, and Fig. 9 shows the results of Experiment No. 1 graphically, together with a diagrammatic representation of the stages of development of the inclusion. It will be seen that the virus appears in the fluid after a latent period of

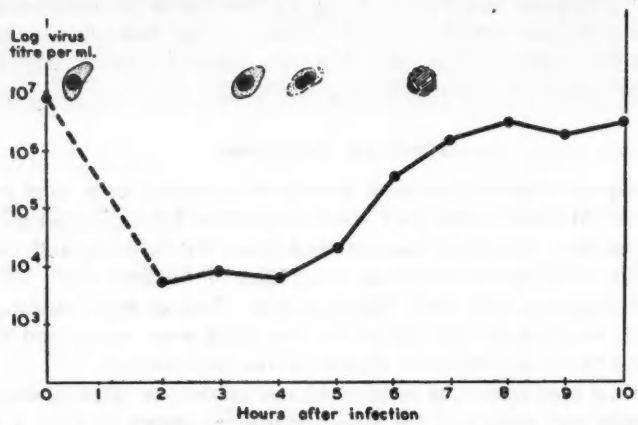

FIG. 9. Increase in infectivity in the culture fluid of monkey kidney tissue cultures infected with Type 1 poliomyelitis.

TABLE I

THE TITER OF VIRUS (CPD_{50}/ML.) IN THE NUTRIENT FLUID OF COVER SLIP CULTURES INFECTED WITH TYPE 1 POLIOMYELITIS VIRUS

Time after infection, hr.	Experiment number		
	1	2	3
0	6.9*	7.2*	7.3*
1	—	—	2.3
2	3.7	3.3	2.5
3	3.9	3.5	2.1
4	3.7	3.7	2.5
5	4.3	4.3	4.1
6	6.0	5.9	5.9
7	6.7	6.5	−5.7
8	7.0	—	—
9	6.7	6.3	5.7 (9½ hr.)
10	7.0	6.1	

* *In experiments Nos. 1 and 2 the cultures were washed after one hour, in No. 3 after one-half hour.*

four to five hours. Fogh (8) has recently reported very similar results for the latent period in monkey kidney cells infected with a large dose of poliomyelitis virus.

The only detailed study of the relationship between inclusion body development and the release of virus was made, as described, with the Mahoney strain. However, as part of a program of isolation of poliomyelitis viruses in the diagnosis of poliomyelitis, cover slip cultures were examined for the presence of inclusions on many occasions. Freshly isolated strains appear to produce cytoplasmic inclusions similar to those described for the Mahoney strain. Furthermore, infection of cultures with stock strains of Types 2 and 3 has been found associated with development of apparently identical cytoplasmic inclusions.

Discussion

The present observations suggest that the large cytoplasmic inclusion body seen in poliomyelitis infected cells of monkey kidney tissue cultures is associated with the production of virus. No similar inclusions have ever been seen in normal monkey kidney tissue cultures, but they appear regularly in cells infected with all three types of poliomyelitis virus. The inclusion body probably appears in the cells before the release of virus into the culture fluid, and the cells disintegrate by vacuolation of the cytoplasm at about the same time as virus appears in the culture fluid. The "central rigid area" seen in living infected cells with the phase contrast microscope (3, 12) is

presumably the structure that appears as an inclusion in our fixed preparations. Lwoff *et al.* (12) have described an essentially similar sequence of events in single monkey kidney cells studied with the phase contrast microscope. Their observations made on single cells show conclusively that the cellular changes precede the release of virus from the cell. The inclusion found in human tonsillar fibroblasts appears to undergo a similar evolution, but 30 to 48 hr. elapse before the cell disintegrates (2, 3).

No similar inclusion body has been described in human tissues from patients with poliomyelitis. It is perhaps of interest that we have not found inclusions in cultures of a human carcinoma cell, strain HeLa, infected with poliomyelitis virus. Ackerman *et al.* (1) also found no inclusions in HeLa cells infected with Type 3 poliomyelitis virus.

An intranuclear inclusion has been described in poliomyelitis infection of nerve cells in man and monkeys (5, 6, 9, 16). These inclusions are small and eosinophilic and are almost certainly identical with those that we have seen in monkey kidney cells (Fig. 5). They appear later than the cytoplasmic inclusion body, but only in a few of the cells. This may be due to the method of fixation, since the intranuclear inclusions in monkey and human neurones are only seen irregularly unless the tissues are specially fixed (9). It is probable that the lesions are a result of virus action causing degeneration of the nucleus. This view is supported by the findings of Kaplan and Melnick (10) that infective virus appears earlier in the cytoplasm than the nucleus of mouse brain cells infected with Type 2 virus. Klöne (11) has described the first change in monkey kidney cells infected with Type 3 virus, and observed under the phase contrast microscope, as an enlargement and prominent appearance of the mitochondria, similar to that seen in cells undergoing active metabolism.

The presence of mitochondria in the inclusion now described suggests that the energy yielding processes known to be associated with those structures may be concerned with the development of virus. There is, however, no proof as yet that the inclusion body actually contains developing virus. These inclusions are not specific for poliomyelitis, as similar structures occur in cultures infected with Coxsackie Group B or "Orphan" viruses.

Acknowledgments

Our thanks are due to Dr. S. H. Bensley for advice on the interpretation of preparations stained for mitochondria. The microphotographs were all taken by Mr. H. F. Whitaker of the Department of Anatomy, University of Toronto.

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EVALUATION OF THE ENERGY GAINED BY PSEUDOMONAS AERUGINOSA DURING THE OXIDATION OF GLUCOSE TO 2-KETOGLUCONATE[1]

By J. J. R. Campbell, T. Ramakrishnan,[2] A. G. Linnes, and B. A. Eagles

Abstract

Cell extracts of *Pseudomonas aeruginosa* were shown to oxidize glucose with the accumulation of 2-ketogluconate. The theoretical amount of oxygen was taken up during the reaction and no added hydrogen acceptors were necessary. With glucose as substrate these extracts did not form high energy phosphate nor did they reduce diphosphopyridine nucleotide or triphosphopyridine nucleotide. Almost identical amounts of growth were obtained when limiting equimolar amounts of glucose, gluconate, or 2-ketogluconate were compared as sole sources of carbon. It was concluded that *P. aeruginosa* 9027 does not gain energy during the oxidative steps glucose ⟶ gluconate ⟶ 2-ketogluconate.

Introduction

Earlier studies by Stokes and Campbell (10) established that *Pseudomonas aeruginosa* 9027 oxidizes glucose by way of gluconic and 2-ketogluconic acids. Dried cell preparations were able to carry out these oxidations at a normal rate in the presence of sodium fluoride, and in addition adenosine triphosphate (ATP) did not stimulate the reaction, thus indicating that phosphorylation of the substrate was not involved. Additional data by Campbell and Norris (2) showed that glucose-6-phosphate and other members of the conventional Embden–Meyerhof pathway were not present during the oxidation of glucose, thus confirming the major role which the nonphosphorylated intermediates play in the oxidation of glucose. Using a purified enzyme system, Ramakrishnan and Campbell (9) found that the gluconic dehydrogenase of *Pseudomonas aeruginosa* oxidized gluconate to 2-ketogluconate without either producing high energy phosphate or being stimulated by its presence. In preliminary growth experiments Campbell, Linnes, and Eagles (1) showed that *P. aeruginosa* achieved equal amounts of growth when limiting equimolar amounts of glucose, gluconate, or 2-ketogluconate were used as the sole source of energy. The present experiments were undertaken in an effort to consolidate the information available and to introduce new criteria for determining whether or not energy is gained by *P. aeruginosa* 9027 when glucose or gluconate is oxidized to 2-ketogluconate.

Materials and Methods

For experiments requiring a cell extract *Pseudomonas aeruginosa* ATCC 9027 was grown in the glucose – ammonium phosphate medium of Norris and Campbell (7) at 30° C. for 20 hr. in Roux flasks. The harvested cells were

[1]*Manuscript received October 1, 1955.*
Contribution from the Department of Dairying, Faculty of Agriculture, The University of British Columbia, Vancouver, British Columbia. This work was carried out under a grant from the National Research Council of Canada.
[2]*Recipient of a Fellowship from the Canada Department of Trade and Commerce under the Colombo Plan.*

washed once, disintegrated in a 10 kc. sonic oscillator, and centrifuged for one hour at 28,000 *g*. Diphosphopyridine nucleotide (DPN) or triphosphopyridine nucleotide (TPN) reduction was measured in a DU spectrophotometer at 340 mμ. The production of ATP was measured by the method of Kornberg (5). The reaction mixture contained glucose (5.0 μM.) 0.2 ml., $MgCl_2$ (15 μM.) 0.1 ml., hexokinase from Pabst laboratories (10 mgm. per ml.) 0.05 ml., glucose-6-phosphate dehydrogenase from Sigma Chemical Co. (3 mgm. per ml.) 0.1 ml., TPN from Pabst Laboratories (2 mgm. per ml.) 0.1 ml., adenylic acid (AMP) (50 μM. per ml.) 0.1 ml., and sonic extract of *P. aeruginosa* 0.1 ml. The optical density was measured at 340 mμ. A control experiment was conducted with 0.1 ml. ATP (50 μM. per ml.) in place of the sonic extract. The absence of adenosine triphosphatase in the above reaction mixture was determined by omitting glucose and analyzing for increase in inorganic phosphorus by the method of Lowry and Lopez (6). There was no increase in inorganic phosphate on incubating the mixture at 30° C. for 40 min.

The presence of DPNH oxidase was checked by noting the decrease in optical density, if any, when 0.1 ml. of the sonic extract was added to a mixture containing 0.1 ml. (2 mgm. per ml.) of DPNH. The DPNH was prepared by reducing DPN in the presence of alcohol dehydrogenase and a limiting concentration of alcohol by the method of Racker (8).

TPNH was prepared by the action of the *iso*citric dehydrogenase of *P. aeruginosa* in the presence of a limiting concentration of *iso*citrate.

In the experiments designed to compare the ability of the three substrates to support growth the organism was cultured on a medium containing 0.3% ammonium dihydrogen phosphate, 0.3% dipotassium hydrogen phosphate, 2 p.p.m. Fe^{++} added as ferrous sulphate heptahydrate, 0.1% magnesium sulphate heptahydrate, 0.02% Tween 80, and a carbon source. The magnesium sulphate and carbon source were added aseptically after sterilization. All cultures were grown in 25 ml. quantities in rubber-stoppered 250 ml. Erlenmeyer flasks at 30° C. with constant shaking. The flasks had been chemically cleaned with hot dichromate cleaning solution, rinsed in tap water at least 12 times, filled with distilled water, and autoclaved at 15 lb. pressure for 15 min. Growth was measured by aseptically removing a 2 ml. aliquot, diluting it to 10 ml., and observing the turbidity in a Fisher Electrophotometer using a 450 mμ filter.

Bacterial protein was determined by the biuret test of Hiller *et al.* (4) after precipitation of the protein with an equal volume of 10% trichloroacetic acid. Paper chromatography of the substrates was achieved by the method of Norris and Campbell (7).

Experimental

In agreement with the results obtained with dried cells (10) it was found that extracts, prepared by treatment of the cells with the sonic oscillator, oxidized glucose or gluconate quantitatively to 2-ketogluconate (Fig. 1) and

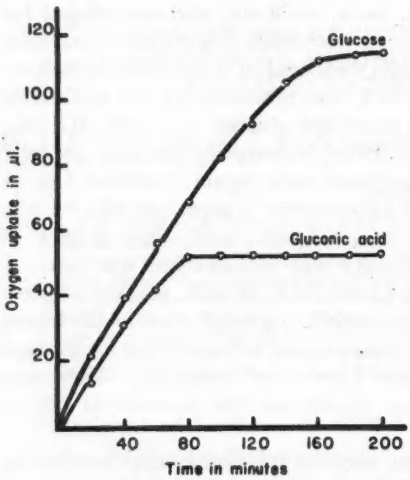

FIG. 1. Oxidation of glucose and gluconate by cell extracts of *P. aeruginosa*. Each Warburg vessel contained; 1.0 ml. of cell extract, 1.0 mM. phosphate buffer of pH 6.0, 5 µM. substrate, 0.15 ml. 20% KOH, and water to 3.15 ml.

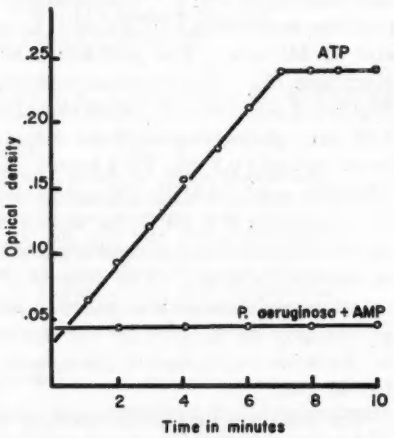

FIG. 2. Formation of high energy phosphate during glucose oxidation as measured by TPN reduction in the presence of hexokinase and glucose-6-phosphate dehydrogenase. The first cuvette contained 5 µM. glucose, 15 µM. magnesium chloride, 1 mgm. hexokinase, 0.3 mgm. glucose-6-phosphate dehydrogenase, 0.2 µM. TPN, 5 µM. ATP, 1 mM. phosphate buffer pH 7.0, and water to 3.0 ml. In the second cuvette 0.1 ml. of sonic extract of *P. aeruginosa* and 5 µM. of AMP replaced ATP.

that sodium fluoride at a final concentration of $M/18$ did not interfere with these oxidations. The theoretical amount of oxygen required for the conversion of glucose or gluconate to 2-ketogluconate was taken up and 2-ketogluconate was identified as the end product by paper chromatography (7). Since the series of oxidations stopped completely at 2-ketogluconate and since systems necessary for the transfer of hydrogen from substrate to oxygen are present it is possible to use these extracts for determining the transfer of energy coincident with the oxidation of glucose or gluconate to 2-ketogluconate.

In an attempt to determine whether or not the oxidation of glucose or gluconate to 2-ketogluconate by these sonic extracts resulted in the formation of high energy phosphate, the technique of Kornberg (5) was employed. The reaction mixture contained hexokinase, glucose-6-phosphate dehydrogenase, adenosine monophosphate (AMP), magnesium (Mg), TPN, and the sonic extract of *P. aeruginosa*. Under these conditions there was no reduction of TPN and, therefore, no ATP was generated by the oxidation of glucose to 2-ketogluconate. Substitution of ATP for the AMP and *P. aeruginosa* extract resulted in rapid reduction of TPN (Fig. 2). Since the enzymes and the sonic extract were free of adenosine triphosphatase and TPN oxidase it

would appear that the experiment rules out the possibility that high energy phosphate is formed during the oxidation of either glucose or gluconate to 2-ketogluconate.

Friedkin and Lehninger (3) have shown that esterification of inorganic phosphate occurs during the oxidation of reduced pyridine nucleotides. It therefore follows that if these coenzymes participate in either the oxidation of glucose to gluconate or of gluconate to 2-ketogluconate the subsequent oxidation of the reduced coenzyme should result in a gain of energy to the system. However, it was found that DPN or TPN did not act as hydrogen acceptors during the oxidation of glucose or gluconate by sonic extracts of *P. aeruginosa* and further, these extracts did not contain either a DPNH or TPNH oxidase.

Although DPN and TPN are not reduced, it is possible that other coenzymes are reduced and their reoxidation might result in the formation of high energy bonds. To test this possibility it was decided to compare the relative ability of glucose, gluconate, and 2-ketogluconate to support growth when they were present in limiting concentration as the sole source of carbon. In order to determine whether or not the amount of growth was a linear function of the concentration of carbon source, cultures of *P. aeruginosa* were grown in 25 ml. quantities of the basal medium containing various concentrations of glucose. The medium was dispensed in 250 ml. Erlenmeyers, incubation was carried out on a shaking machine at 30° C., and growth measurements were made at 24 hr. (Fig. 3) (Table I). The limiting concentration of glucose chosen for further work was 0.2%. The corresponding amount of gluconate was added as gluconolactone (0.198%). The 2-ketogluconate was dissolved as Ca (2-ketogluconate)$_2$. $3H_2O$ (0.266%) and the calcium removed with an equimolar amount of sodium sulphate. The rates of growth with the three

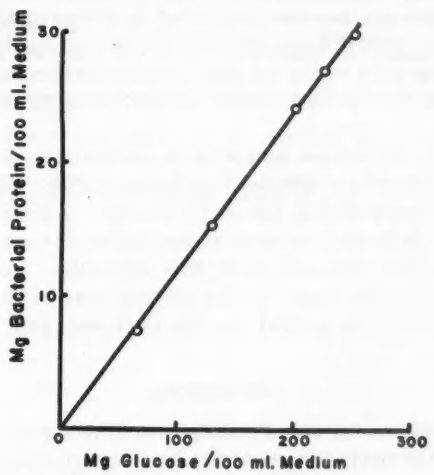

FIG. 3. Growth of *P. aeruginosa* as a function of glucose concentration.

TABLE I

GROWTH AS A FUNCTION OF GLUCOSE CONCENTRATION

Conc. of glucose, %	Growth		Relative conc. of glucose	Relative amount of growth
	Light transmission, %*	Protein/100 ml. med. mgm.		
0.0625	87.0	7.6	1.0	1.0
0.125	76.1	15.1	2.0	1.99
0.175	68.0	22.1	2.8	2.91
0.20	65.5	24.0	3.2	3.16
0.225	61.5	27.0	3.6	3.56
0.250	58.5	29.7	4.0	3.91

* *Culture diluted 1/5.*

TABLE II

GROWTH RESPONSE OF *P. aeruginosa* TO LIMITING EQUIMOLAR AMOUNTS OF GLUCOSE, GLUCONATE, OR 2-KETOGLUCONATE

Substrate	Light transmission, %*	Time to reach maximum growth, hr.
Glucose	64.25	17
Gluconate	64.50	16
2-Ketogluconate	64.00	22

* *Culture diluted 1/5.*

substrates differed. Gluconate characteristically gave the fastest growth while 2-ketogluconate supported the slowest growth. The values taken for purposes of comparison are the points of maxium turbidity (Table II). Concentration of the growth medium at the end of the experiment and analysis by paper chromatography indicated that no substrate remained in any of the flasks. The complete experiment was repeated nine times with essentially the same results.

It is evident that equimolar amounts of the three compounds supported equal growth. It must be concluded that even growing cells of this organism do not gain any energy during the steps glucose $\longrightarrow$ gluconate $\longrightarrow$ 2-ketogluconate. These data also serve to re-emphasize the major role that this pathway plays in the metabolism of this organism. For it would seem reasonable to expect that if part of the glucose were oxidized by any other pathway energy would be gained by the organism and increased growth would result.

Discussion

The fact that sonic extracts of *P. aeruginosa* have a good ability to oxidize glucose and that the oxidation stops at the 2-ketogluconate stage makes it possible to study this over-all reaction without further fractionation of the

extract. Gluconate has been shown to be an intermediate in this oxidation and so the use of glucose as substrate should allow any effects produced by the glucose ⟶ gluconate step plus those produced by the oxidation of gluconate to 2-ketogluconate to be observed. Admittedly some of the techniques used show only that specific systems are missing and, therefore, that no energy can be expected from this source. This criticism applies to the observation that neither DPN nor TPN are reduced with glucose as substrate. To a lesser degree it applies to the observation that high energy phosphate is not transferred to the adenylic acid system as a result of the oxidation. However, these observations plus the fact that the growth experiments show that the organisms obtained the same amount of energy from equimolar amounts of glucose, gluconate, or 2-ketogluconate lead us to conclude that the oxidation of glucose to 2-ketogluconate by way of gluconate is not linked with any energy–transferring system.

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PHASE VARIATION OF THE NINE MILE AND OTHER STRAINS OF RICKETTSIA BURNETI[1]

By M. G. P. Stoker and P. Fiset[2]

Abstract

The classical Nine Mile strain of *Rickettsia burneti*, which had been maintained in guinea pigs since first isolation, was adapted to growth in yolk sacs. Despite abundant rickettsiae, antigens prepared from each of the first seven yolk sac passages failed to react in complement fixation tests with homologous Q fever antisera. The antisera did react, however, with antigens prepared from the eighth and subsequent passages. This change in behavior during egg adaptation, which resembles that found previously with freshly isolated strains, is termed "phase variation". Rickettsiae in an early stage of egg adaptation which fail to fix complement with antisera are called Phase 1; after further egg adaptation and the development of ability to react with antiserum, they are termed Phase 2. Strains in Phase 2 rapidly reverted to Phase 1 after inoculation into guinea pigs, mice, or hamsters, and naturally occurring strains from a sheep's placenta, cows' milk, and ticks were also found to be in Phase 1. It is possible that all strains used for preparing stock complement fixing antigens are laboratory variants in Phase 2 obtained through multiple yolk sac passages.

Introduction

Several authors have reported that strains of *Rickettsia burneti* from different sources vary in their serological behavior (1, 7, 12). Those which have been studied may be broadly divided into two groups according to their activity in complement fixation tests: Group 1, strains such as Henzerling and Nine Mile, which although not identical (11) yield antigens which react well with guinea pig sera prepared against homologous and heterologous strains of *R. burneti* and with antisera from a wide range of naturally infected animals; Group 2, strains such as the Panama (2) and Konitzer (1), which unless used in high concentration react poorly with all antisera, including those which show high titers with Henzerling or Nine Mile antigens.

Two strains of *R. burneti* isolated in Great Britain appeared at first to be in Group 2 because antigens prepared from early yolk sac passages, although rich in rickettsiae, failed to react with homologous or heterologous antisera. After further egg adaptation, however, a change occurred and antigens made from the fifth and subsequent yolk sac passages resembled those made from the Henzerling and Nine Mile strains and reacted with a wide range of Q fever antisera (9, 10). This variation, which was not due to an increase in numbers of rickettsiae in the antigens, was confined to their behavior in fixation of complement with antibody. It did not extend to their agglutinability, either by Q fever antibody itself, or, after absorption of Q fever antibody, by antiglobulin serum (3).

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A similar variation during egg adaptation was found by other workers ((6) and Berge, personal communication) and this raised the possibility that it might be of general application to *R. burneti*, and might account for some of the differences between strains in Groups 1 and 2 described above, because the strains were not compared after an equal number of egg passages. If so, an admittedly good complement fixing strain, such as the classical Nine Mile strain, would react poorly with antisera when the strain was in the early stages of egg adaptation.

The Nine Mile strain was isolated from ticks in Montana (5) and was later adapted to growth in yolk sacs (4). Since it yields antigens which react with antisera against many different strains of *R. burneti*, the Nine Mile strain is widely used for the routine diagnosis of Q fever. Most lines of this strain have already undergone numerous egg passages and are unsuitable for study of the early stages of egg adaptation; but one line of the Nine Mile strain has been maintained at the Rocky Mountain Laboratory, Montana, entirely by guinea pig passage.

We have studied the variability of this guinea pig adapted strain on passage in yolk sacs, and of other strains freshly isolated from ticks, sheep, and cows. We have also reversed the variation by passage of the egg adapted strains in animals, and have investigated the antibody response of man and guinea pigs with antigens prepared from strains in different stages of the variation.

For convenience we refer to strains as being in Phase 1, when in the early stages of egg adaptation they react poorly with complement fixing antibody; and in Phase 2, when, after further passage in yolk sacs, they react well with such antibody. The phenomenon in general is referred to as "phase variation".

Methods and Materials

For passage of strains in animals, fully grown guinea pigs, weighing 300 to 500 gm., were inoculated peritoneally with 2 ml. quantities of the appropriate suspensions of yolk sac, or of spleen from previously inoculated animals. Hamsters were similarly inoculated with 1 ml. and mice with 0.25 ml. of the suspensions. Ten per cent horse serum in tryptic digest broth was used as diluent throughout. The methods of yolk sac inoculation, passage in fertile hens' eggs, and preparation of antigens from yolk sac suspensions by ether extraction and differential centrifugation were those already described (10).

Serological Tests

Human antisera were obtained from patients, including the patient from whom the Christie strain had originally been isolated, at varying times after an attack of Q fever; guinea pig antisera were obtained by cardiac puncture. After separation from the clot, antisera were stored at −20° C., and were heated to 60° C. before testing.

Complement fixation tests were carried out by the standard technique used in this laboratory (11). Most of the antigens were tested by titrating increasing doubling dilutions of the antigen with increasing doubling dilutions of

homologous antiserum in the presence of two minimal haemolytic doses of complement. Such titrations with graded concentrations of antigen as well as of antiserum, are referred to as "chessboard titrations". In Figs. 1, 2, and 3 these chessboard titrations are recorded pictorially. The four antigen dilutions on the vertical scales range from 1 : 10 to 1 : 80. The antiserum dilutions on the horizontal scales start at 1 : 10 and finish at 1 : 1280 for the Nine Mile antiserum and at 1 : 320 for the Christie antiserum.

Strains of R. burneti

The Christie strain was described in previous communications (9, 10). The Nine Mile strain, as used for routine preparation of diagnostic antigen, was received as lyophilized yolk sac suspension from Dr. Cox of the Lederle Laboratories in 1949, after 50 or more egg passages. This egg adapted line, which reacts well with a variety of Q fever antisera, is referred to as the NM (Led) strain.

The guinea pig adapted line of the Nine Mile strain was received from Dr. Lackman of the Rocky Mountain Laboratory as lyophilized spleen suspension from the 307th guinea pig passage. This line, which had never been passaged in eggs, is referred to as the NM (Rocky) strain.

The source of other, freshly isolated, strains is given below.

Standardization of Rickettsial Concentration

When comparing the behavior of various rickettsial suspensions as antigens, it is obviously important that the concentration of organisms in each suspension should be approximately the same. Since the organisms do not obviously vary in size, and, judging by direct and electron microscopy, there is practically no extraneous material in the final preparation, the antigens were matched by opacity in a photoelectric densitometer with an NM (Led) strain antigen containing 31.3 mgm. of nitrogen per 100 ml. as a standard.

To find whether antigens matched by this method contained approximately equal numbers of rickettsiae, direct counts were made of the organisms in eight selected antigens using the electron microscope technique of Williams and Backus (13). The numbers of rickettsiae in the matched antigens were found to lie between 2×10^{13} and 3×10^{13} per ml.; differences of this order did not affect the significance of results obtained in the complement fixation tests.

Results

Variation of the Nine Mile Strain

The lyophilized material from the 307th guinea pig passage of the NM (Rocky) strain was inoculated into guinea pigs; spleens taken during the febrile period in this 308th guinea pig passage were then inoculated as 10% suspensions into yolk sacs of fertile hens' eggs. The embryos died 11 days later and the smears from the yolk sacs showed a profuse growth of rickettsiae. Antigens prepared from these yolk sacs were tested for fixation of complement, in chessboard titrations with homologous guinea pig antiserum taken 21 days

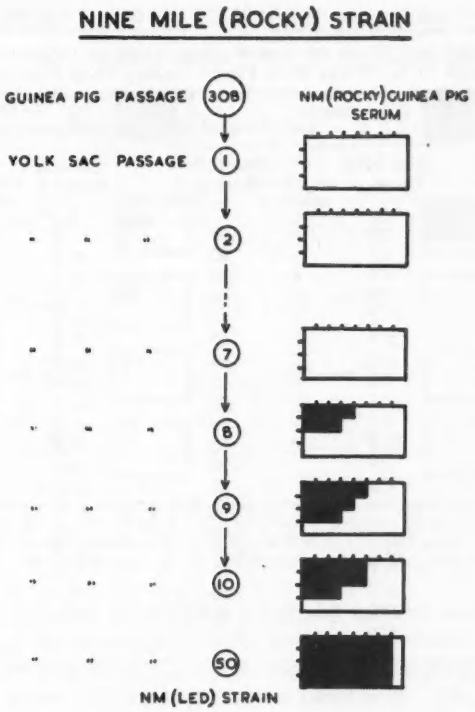

FIG. 1. The effect of successive yolk sac passage on antigens from the Nine Mile (Rocky) guinea pig adapted strain of *R. burneti*, tested by complement fixation with homologous guinea pig antiserum. (For explanation of the diagrams, see Methods, Serological Tests).

after inoculation with the NM (Rocky) strain in the form of infected guinea pig spleen suspension. Stock antigen prepared from the egg adapted NM (Led) strain was also tested with the same antiserum.

NM (Rocky) antigen prepared from the first egg passage failed completely to react with the antiserum in the dilutions tested, whereas an antiserum titer of 1 : 640 was obtained with NM (Led) antigen (Fig. 1).

It was assumed that NM (Rocky) strain was in Phase 1 and it was passaged serially in yolk sacs, using as inoculum a 1/1000 suspension of yolk sac from the previous passage. Antigen was prepared from four or five yolk sacs at each passage and tested with the same homologous guinea pig antiserum. At the eighth passage the antigen reacted with the antiserum and in subsequent passages the degree of complement fixation increased, but was not as marked as that shown by the NM (Led) antigen prepared from the 50th or greater yolk sac passage (Table I). In a later experiment, however, yolk sac passages were carried out with a more concentrated inoculum, diluted 1 in 10 instead

TABLE I

COMPLEMENT FIXATION TITERS OF HUMAN SERA TAKEN AT LONG INTERVALS AFTER ATTACKS OF Q FEVER, WITH PHASE 1 AND PHASE 2 ANTIGENS

Serum obtained (years after illness)	Reciprocal of titer with antigens from			
	Nine Mile Phase 1	Nine Mile Phase 2	Christie Phase 1	Christie Phase 2
1	5	160	5	160
1	<5	80	< 5	80
1	<5	20	< 5	10
2	<5	40	< 5	20
2	<5	20	< 5	10
2	<5	10	< 5	< 5
3	<5	40	< 5	20
3	<5	< 5	< 5	< 5
4	<5	80	< 5	20
4	<5	80	< 5	20
4	<5	40	< 5	20
4	<5	20	<10	10
4	<5	20	<10	10
4	<5	<10	< 5	< 5
4	NT	<10	< 5	< 5
4	NT	< 5	< 5	< 5
12	NT	40	10	40
12	NT	40	< 5	20
14	NT	40	< 5	10
14	NT	< 5	< 5	< 5

NT = Not tested.

of 1 in 1000, starting at the sixth passage in the original series. This led, from the eighth passage, to the development of antigens closely resembling those prepared from the NM (Led) strain.

When the antigens were tested with other antisera, for example, from guinea pigs inoculated with NM (Led) strain, or with Christie strain human antiserum, similar results were obtained.

Thus it is clear that the Nine Mile strain, like the Christie strain, is subject to the variation and changes from Phase 1 to Phase 2 when passaged in yolk sacs.

Reversal of the Variation

All the strains found to be in Phase 1 on first growth in eggs had previously been passaged in guinea pigs. Attempts were therefore made to reverse the variation by inoculation of egg adapted Phase 2 strains into guinea pigs.

Yolk sac suspensions of the Christie strain in its ninth egg passage, and of the NM (Led) strain in its 50th (or more) passage, were inoculated into guinea pigs. At the height of the resulting febrile response the guinea pigs were killed and 10% suspensions of spleens were used for further passage. Five guinea pig passages of the two strains were carried out.

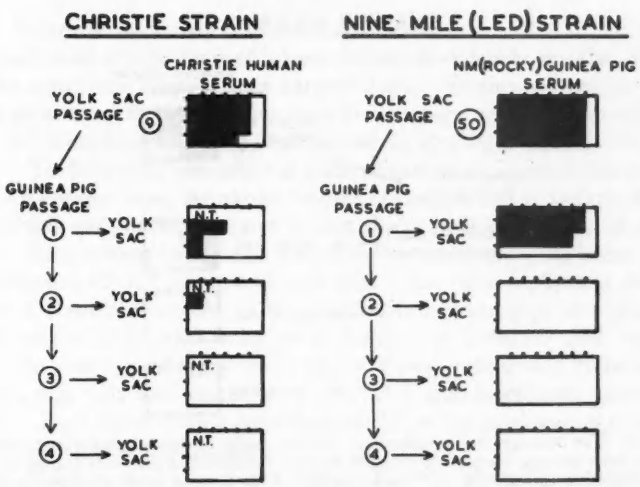

FIG. 2. The effect of successive guinea pig passage on antigens from the egg adapted Christie strain of *R. burneti*, tested by complement fixation with human convalescent serum; and from the egg adapted Nine Mile (Led) strain, tested with homologous guinea pig antiserum. (For explanation of the diagrams, see Methods, Serological Tests).

It is difficult to make satisfactory rickettsial suspensions from guinea pig tissues for testing the complement fixing activity of the strains. Accordingly, some of the spleen suspension at each passage was used to inoculate yolk sacs, from which antigens were prepared in the usual way. The validity of this procedure depends on the assumption that if the strains had reverted to Phase 1 in the guinea pigs, one yolk sac passage would not be sufficient for reversion to Phase 2. These antigens, derived indirectly from each successive guinea pig passage, were tested with the corresponding Christie human or NM (Rocky) guinea pig antiserum (Fig. 2).

Before inoculation into guinea pigs both the egg adapted strains were in Phase 2 and reacted well with homologous antiserum. When reisolated from the first guinea pig passage at the acute febrile stage and grown in yolk sacs, they were still reacting partly as Phase 2 strains. From the second guinea pig passage onwards, however, the Nine Mile strain had reverted completely to Phase 1 and the Christie strain almost completely.

After five guinea pig passages the NM (Led) strain, now in Phase 1, was readapted to yolk sacs, and seven passages were needed for reversion to Phase 2. Thus the behavior in yolk sacs of the strain after five guinea pig passages was much the same as that found with the NM (Rocky) strain after 308 guinea pig passages.

When material for passage was taken early in the course of infection of the animals, at the height of the febrile response, the change from Phase 2 to Phase 1 occurred at the second or third guinea pig passage. Since *R. burneti* persists for long periods in the tissues of guinea pigs it seemed possible that

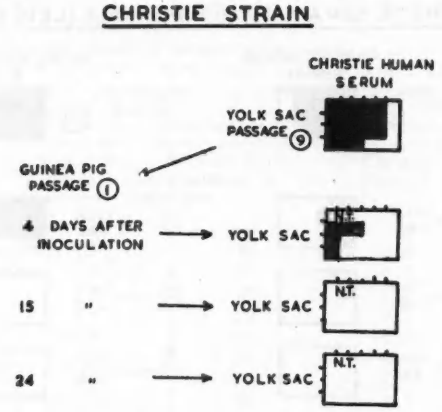

FIG. 3. The change in complement fixation with human convalescent serum by antigens from the egg adapted strain of *R. burneti*, reisolated at intervals during the course of infection in the guinea pig. (For explanation of the diagrams, see Methods, Serological Tests).

the reversion might occur in the first passage if reisolation from the animals was delayed sufficiently. Accordingly Christie ninth-egg-passage material in Phase 2 was inoculated into a number of guinea pigs. At successive intervals after inoculation, animals were killed, and spleen suspensions inoculated into yolk sacs for preparation of antigen. Reversion to Phase 1 occurred in rickettsiae isolated from the guinea pigs 15 days or more after inoculation or 11 days after the acute febrile period (Fig. 3).

Since growth of the rickettsiae in the guinea pig led to reversion from Phase 2 to Phase 1, attempts were made to cause reversion in other animals. Hamsters and mice were inoculated with Christie strain in the ninth egg passage, in Phase 2. Thirty days after inoculation pooled spleen suspensions from hamsters or mice were inoculated into batches of yolk sacs. There was insufficient growth for antigen preparation in the first egg passage from either species of animal, so a further yolk sac passage was carried out. Tests on antigens prepared from these second passage yolk sacs showed that, after growth for 30 days there had been complete reversion to Phase 1 in mice and almost complete reversion to Phase 1 in hamsters. Abinanti and Marmion (personal communication), who for other purposes devised a technique for preparing complement fixing antigens direct from mouse spleens, showed that after mouse inoculation with a Phase 1 strain the spleens contain rickettsiae which are also in Phase 1, demonstrating that the Phase 1 state does occur in the animals themselves and is not limited solely to the early stages of egg adaptation.

We conclude that *R. burneti* exists in Phase 1 when grown in susceptible laboratory animals, and only alters to Phase 2 after fairly prolonged growth in yolk sacs. In this state it reverts to Phase 1 again almost immediately after inoculation into the animals.

State of Naturally Occurring Strains of R. burneti

The biological significance of phase variation depends on the phases in which the organisms exist in their natural hosts. Strains are usually isolated from these sources by inoculation into guinea pigs, mice, or hamsters, and passage in these laboratory animals would in any case convert them into Phase 1. To determine the original state of the strain it would be necessary to make an antigen from the natural source of material, or to isolate the strain by direct yolk sac inoculation and to test the antigenic behavior at an early passage. Strains from ticks, cows, and sheep were tested by the latter method.

A suspension of 371 adult and nymphal ticks (*Haemaphysalis punctata*), collected in Kent and known to be infective for guinea pigs at a dilution of 10^{-4}, was mixed with 1000 units of penicillin per milliliter and inoculated directly into yolk sacs of fertile hens' eggs. Cows' milk known to be infective for guinea pigs was also mixed with penicillin and inoculated directly into yolk sacs. Both these strains grew sufficiently for the preparation of antigens from yolk sacs in the first passage.

A 20% suspension of a placenta from a Kentish sheep, known to contain $10^{4.8}$ guinea pig infective doses of *R. burneti* per gram was filtered through a gradocol membrane with an average pore diameter of 0.7 μ, and the filtrate was inoculated into yolk sacs. There were not sufficient rickettsiae for an antigen preparation in the first yolk sac passage, but antigen was made from the second passage of this strain.

Antigens from first passage yolk sacs of the tick and milk strains and second passage yolk sacs of the sheep placenta strain, tested with high titer NM (Rocky) guinea pig antiserum, were all in Phase 1.

Antibody Response in Man and Guinea Pigs

Smadel and his colleagues (8) showed that antibodies to the poorly complement fixing strains of *R. burneti* eventually appeared in guinea pig sera two months or more after inoculation. Berge and Lennette (1) confirmed this, and found similar antibodies in pools of human sera taken 16 to 28 months, but not in the first six weeks, after natural infection.

Guinea pig and human sera were studied to find whether antigens from strains in Phase 1, although not reacting with antisera taken at the usual time, three to five weeks, after infection, would nevertheless fix complement with antibody appearing much later in the immune response. Guinea pigs inoculated with the NM (Rocky) strain in Phase 1 in the form of guinea pig spleen suspension were bled at frequent intervals during a five month period. The sera were tested with Phase 1 antigens prepared from the second yolk sac passage of the NM (Rocky) strain, or the third yolk sac passage of the Christie strain, and Phase 2 antigens from the 50th (or more) passage of the NM (Led) strain, or the 10th yolk sac passage of the Christie strain.

Instead of chessboard titrations the sera were tested with the optimum antigen dilution of the Phase 2 antigens, as previously determined by chessboard titration with standard antisera (11), and with Phase 1 antigens in an equivalent rickettsial concentration.

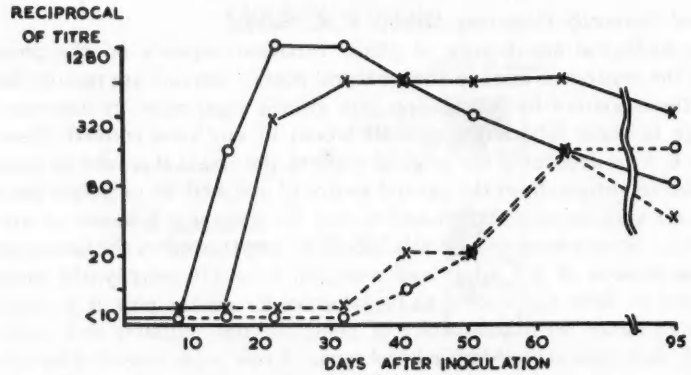

FIG. 4. Phase 1 and Phase 2 complement fixing antibodies in the serum of a guinea pig inoculated with the Nine Mile (Rocky) strain of *R. burneti* in Phase 1. O - - - O Titers with Nine Mile Phase 1 antigen. O——O Titers with Nine Mile Phase 2 antigen. X - - - X Titers with Christie Phase 1 antigen. X——X Titers with Christie Phase 2 antigen.

In all guinea pigs there was a rapid rise of Phase 2 antibody, reaching a peak in the second week after inoculation and then declining, but persisting at a lower level at least until the end of the experiment. Phase 1 antibodies also appeared but were not detectable until five to six weeks after inoculation, increasing until the 10th week and then persisting at about the same level as the Phase 2 antibodies. Fig. 4 exemplifies the antibodies detected by the four antigens.

The only homologous human serum available was that obtained from the patient infected with the Christie strain. Specimens taken up to four months after the illness were tested; no antibody was detectable up to this time with Phase 1 Christie antigen, even in sera with a titer of 1 : 320 with the Phase 2 Christie antigen.

Serum from other patients collected after longer intervals, ranging from 1 to 14 years after attacks of Q fever, were tested with Phase 1 and Phase 2 antigens (Table I). Although most patients had Phase 2 antibody only one had Phase 1 antibody at 1 : 10.

One hundred and eleven sera from a flock of naturally infected sheep were tested with a Phase 1 antigen from the second yolk sac passage of a strain freshly isolated from the placenta of one of the sheep. Only two of the sera reacted at 1 : 10, although 32 sera were positive at 1 : 10 and higher dilutions with antigen prepared from the egg adapted Henzerling strain.

Identification of Phase 1 and Phase 2 Strains with Early and Late Antisera

It is clear that strains in Phase 1 cannot be identified positively as *R. burneti* by testing antigens with stock guinea pig antiserum obtained up to six weeks after inoculation with known strains, or with sera from human patients known to have suffered from Q fever.

Guinea pig serum taken six or more weeks after inoculation containing antibody reacting with Phase 1 strains, however, could be used for confirming that the organisms were in fact *R. burneti.* We subsequently discovered that Phase 1 antibodies develop in the rabbit more quickly and to higher levels than in the guinea pig; late rabbit antiserum taken six weeks or more after inoculation contained Phase 1 antibody in high titer and was especially suitable for identification of Phase 1 strains (Fiset, unpublished). Antigens were therefore tested with both early guinea pig serum and late rabbit serum. The tests confirmed that the organism was *R. burneti,* and also allowed the phase to be identified.

Discussion

Our evidence suggests that the variation in serological behavior first observed during egg passage of two English strains may be widely applicable to *R. burneti.* A guinea pig adapted line of the Nine Mile strain, obtained from the Rocky Mountain Laboratory, was found to be in Phase 1 when first grown in eggs. That is, antigens prepared from early egg passages failed to react with Q fever antisera in complement fixation tests, in marked contrast to the egg adapted line of the Nine Mile strain routinely used for producing diagnostic antigen. After eight yolk sac passages of the guinea pig adapted Nine Mile strain, however, it altered in serological behavior to Phase 2, the antigens then reacting satisfactorily with Q fever antisera. This variation of the strain at the eighth passage resembled that found with the Christie (human) strain at the fifth yolk sac passage, or in an earlier experiment between the sixth and eleventh passages, also that occurring with the MI (milk) strain at about the fifth passage, and Herzberg and Urbach's German strains after the 14th or 15th yolk sac passage (6). Similar changes with other strains have also been found by Berge (personal communication). Three additional strains from cows' milk, sheep's placenta, and ticks were also tested by direct inoculation into yolk sacs without intermediate guinea pig passage; all were in Phase 1.

It is therefore probable that the strains which are used routinely for preparing stock antigens, and which react so well with Q fever antisera, may all be laboratory variants in Phase 2 obtained through multiple yolk sac passage. This view is consistent with the rapid reversion to Phase 1 which occurs when these egg adapted strains are inoculated into either guinea pigs, mice, or hamsters. It should, in fact, be possible to account for some of the previously reported differences between strains as due to variation in the degree of adaptation to growth in yolk sacs; and for the apparent differences in results obtained with the same strain, for example the Dyer strain, when tested in different laboratories (1, 7).

The number of yolk sac passages required for the change to Phase 2 varied from 5 to at least 15. This may depend on inherent differences between strains, possibly connected with the source of the strain. The dilution at which the serial yolk sac passages are carried out might also alter the stage at which the variation becomes detectable. If the change is due to the gradual

replacement of Phase 1 organisms by a Phase 2 variant, passage at high dilutions might eliminate the variant at the early stages, while it was still a rarity. Attempts to maintain strains in Phase 1 by passage at limiting dilutions have not so far been successful owing to difficulties of titrating the organism in yolk sacs, but the change of the NM (Rocky) strain was certainly less complete when it was passaged at 1 : 1000 than when passaged at 1 : 10.

Although complement fixing antibodies to Phase 1 antigens were only occasionally found in low titer in man and sheep, there is a late development of Phase 1 antibodies in guinea pigs, reaching a peak some seven to eight weeks after the Phase 2 antibodies, and resembling the response found by Smadel and his colleagues (8) and Berge and Lennette (1) to antigens from strains of low sensitivity.

In some of its natural hosts and in laboratory animals, therefore, *R. burneti* exists in a form which is unable to react in complement fixation tests with the antibody produced against it, at least in the early phases of the hosts' immune response. Organisms which react well with this antibody are found only after prolonged growth in yolk sacs, where no such antibody is produced, and on reinoculation into animals they rapidly revert to the original state. It is tempting to postulate that the original organisms in Phase 1 have a higher survival value than the Phase 2 variant in animals, in the presence of antibody (or in ticks which may ingest antibody), but not in eggs.

It is also possible, however, that the failure of Phase 1 strains to react with antibody is only an *in vitro* phenomenon, detectable by complement fixation tests alone. Further studies are now in progress on the antigenic differences between Phase 1 and Phase 2 organisms and their reactions with antisera *in vivo* as well as *in vitro*. These may throw further light on the biological significance, if any, of phase variation.

Acknowledgments

Thanks are due to Dr. H. R. Cox of Lederle Laboratories, New York, who supplied the egg adapted Nine Mile strain, and to Dr. D. Lackman of the Rocky Mountain Laboratory, Hamilton, Montana, for the guinea pig adapted Nine Mile strain. We are also indebted to Dr. O'Connor of the Commonwealth Serum Laboratories, Melbourne, and to Dr. B. P. Marmion of the Public Health Laboratory Service (Great Britain) for information about the latter strain. We wish to thank Miss Z. Page for valuable laboratory assistance. The work was supported by a grant from the World Health Organization.

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STUDIES RELATING TO THE FORMATION OF NEUTRALIZING ANTIBODY FOLLOWING THE PROPAGATION OF INFLUENZA AND NEWCASTLE DISEASE VIRUS IN THE BOVINE MAMMARY GLAND[1]

By Chas. A. Mitchell, R. V. L. Walker, and G. L. Bannister

Abstract

The propagation of influenza A (PR 8 strain) and Newcastle disease virus in the bovine mammary gland led to the presence in milk and blood serum of neutralizing antibody. The removal of the gland had a profound effect upon the antibody content of the blood. It is suggested that the major portion of antibody was produced locally in the gland. Whether this results from continuous antigenic stimulation or is a function developed from a fleeting contact with the virus remains unanswered.

Introduction

In previous communications (1, 2) it was demonstrated that influenza A and Newcastle disease viruses instilled into quarters of the mammary gland of cattle resulted in the propagation of virus for several days. The titer rapidly rises, reaches a peak, then descends quickly. About the 12th day the virus can no longer be found. Soon thereafter specific neutralizing antibody is present in the milk of the quarter which had been instilled. A few days later it may be found in the blood serum and somewhat later in the milk of other quarters of the mammary gland. Because it is not economical to use cattle for experimental purposes, an attempt was made to substitute goats. Trials on these indicated that the virus propagated readily in the half of the mammary gland instilled, that neutralizing antibody formed, and that in general the response of goats was similar to that of cattle. It was observed that there was some difference in the behavior of the immune mechanism following the stimulus brought about by influenza virus and Newcastle disease virus. In the former, antibody rose following propagation, finally levelled off, and with little decline persisted for many months—perhaps might continue to persist for years. In the case of stimulus from Newcastle disease virus, however, the antibody level was maintained for approximately five months when it began to decline and finally almost disappeared. It was, therefore, apparent that the propagation of influenza virus resulted in the development of neutralizing antibody of high level for long periods of time, perhaps permanently, whereas Newcastle disease virus induced a temporary stimulation of antibody production.

One of the interesting features relating to a long term development of antibody is whether a constant antigenic stimulus is necessary or if the rather

[1]*Manuscript received October 6, 1955.*
Contribution from the Animal Pathology Division, Canada Department of Agriculture, Animal Diseases Research Institute, Hull, Quebec.

fleeting experience with the virus is sufficient to maintain production. Because of the difference in the behavior of the host to the two viruses, both of which propagated in the gland, it was felt that the removal of the infected half might throw some light on the site of formation and perhaps the stimulus required for the production of neutralizing antibody. It was, therefore, determined to instill Newcastle disease and influenza viruses into the mammary glands of suitable goats. After approximately 40 days, when the development of antibody was well established, it was planned to remove in one instance the whole gland and in the other the instilled half only and determine the effect, if any, on antibody production.

Materials and Methods

Material Used for Instillation

The virus of influenza A (PR 8 strain) was propagated in nine-day-old developing chick embryos. When the embryos were almost dead the eggs were opened and the clear fluids harvested. This usually titered 10^{-7}. A dose of 2.0 cc. of this fluid was introduced into the lactiferous sinus of the gland.

The Canadian Twiss strain of Newcastle disease was employed. It was propagated in nine-day-old embryos. When the embryos died, the clear fluids were collected. This material, which usually titered 10^{-8}, was used for instillation.

Methods of Instillation

Healthy goats that were in active lactation were used as experimental animals. The milk was removed from the mammary gland, then by the use of a teat tube and a hypodermic syringe 2 cc. of the appropriate clear fluid carrying the virus was instilled into the lactiferous sinus without injury to tissue. Samples of milk and blood were collected daily and the content of virus determined by means of chick embryo inoculation and the haemagglutination test.

Surgical Removal of the Gland

The virus was present in the milk of the half instilled for approximately 10 days after which it disappeared and neutralizing antibodies were soon found present. Approximately forty days after the instillation of virus and when antibody both in the milk and blood serum had reached a substantial level, the whole gland or that half of the gland which had been instilled was removed surgically. The appropriate tissues were ground in a colloid mill and an attempt made to obtain free virus by means of the inoculation of chick embryos and appropriate animals. At no time was free virus found. Figs. 1 to 6 indicate the details concerning the propagation of virus and the titer of antibody before and after the surgical removal of a part or the whole of the gland.

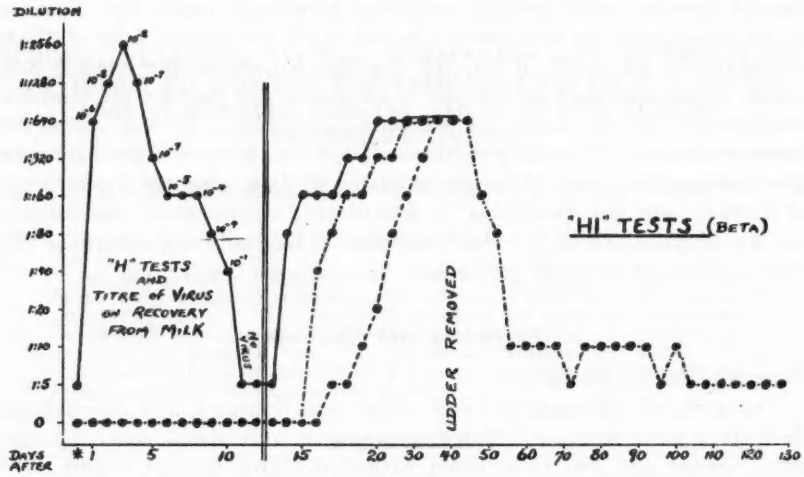

FIG. 1. Goat B22185, 2.0 cc. Newcastle disease virus instilled in right half of udder.
* Day of inoculation.
——— Milk from inoculated (right) half of udder.
- - - - - - - - Milk from left (normal) half of udder.
- . - . - . - . Blood serum.

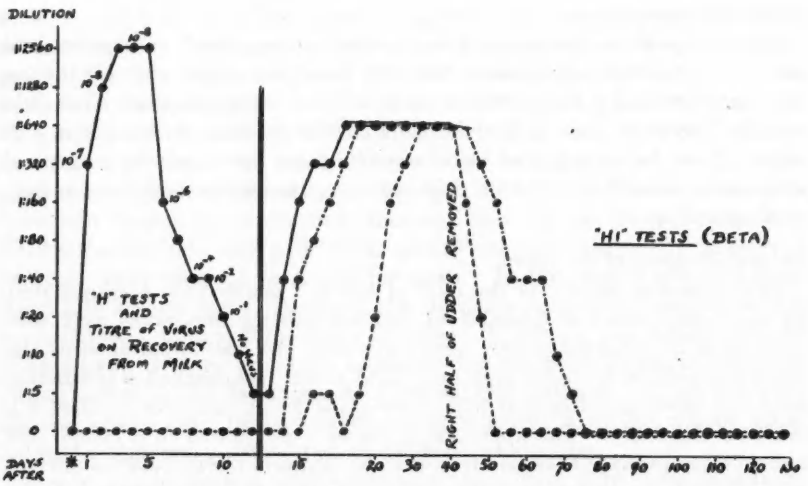

FIG. 2. Goat B22179, 2.0 cc. Newcastle disease virus instilled in right half of udder.
* Day of inoculation.
——— Milk from inoculated (right) half of udder.
- - - - - - - - Milk from left (normal) half of udder.
- . - . - . - . Blood serum.

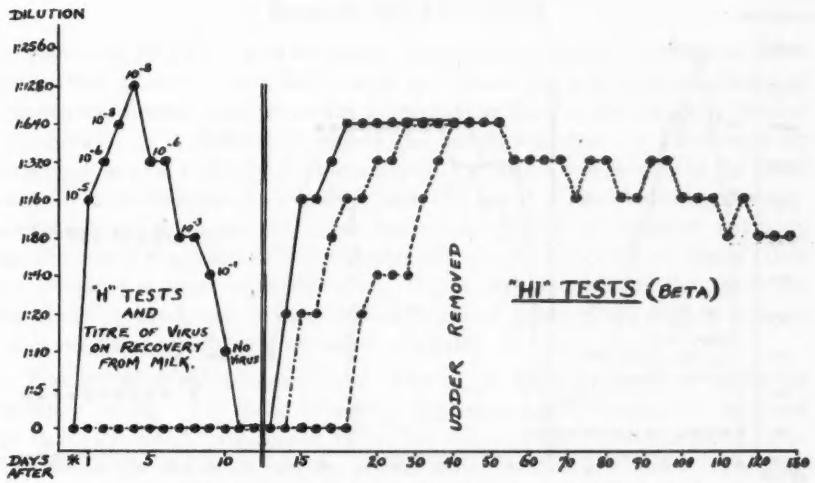

FIG. 3. Goat B22178, 2.0 cc. influenza "A" virus instilled in right half of udder.

* Day of inoculation.
———— Milk from inoculated (right) half of udder.
- - - - - - - - Milk from left (normal) half of udder.
- . - . - . - . Blood serum.

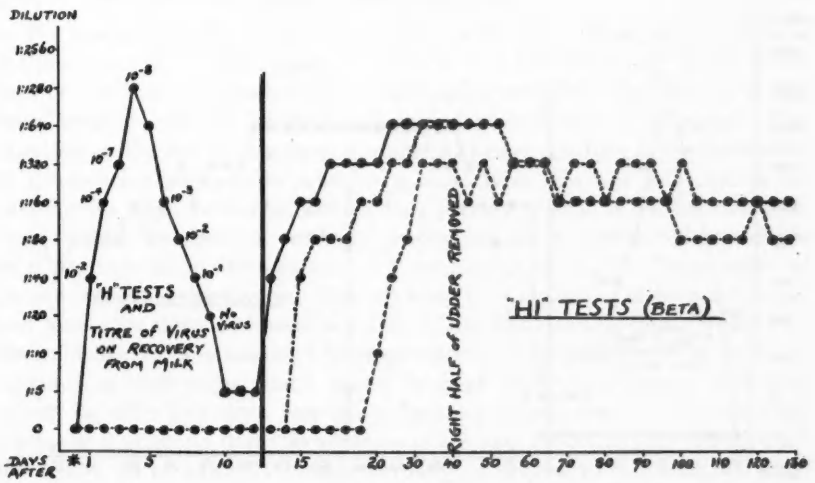

FIG. 4. Goat B22182, 2.0 cc. influenza "A" virus instilled in right half of udder.

* Day of inoculation.
———— Milk from inoculated (right) half of udder.
- - - - - - - - Milk from left (normal) half of udder.
- . - . - . - . Blood serum.

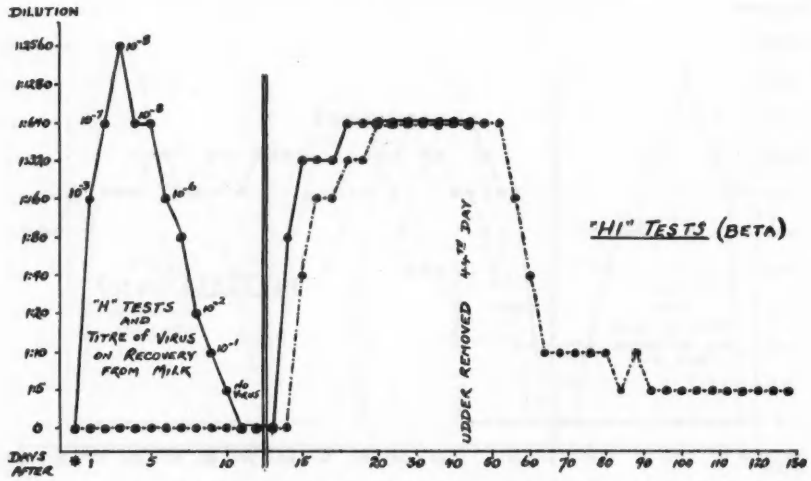

FIG. 5. Goat B22186, 2.0 cc. Newcastle disease virus instilled in right half of udder.
* Day of inoculation.
———— Milk from inoculated (right) half of udder.
- - - - - - - Blood serum.

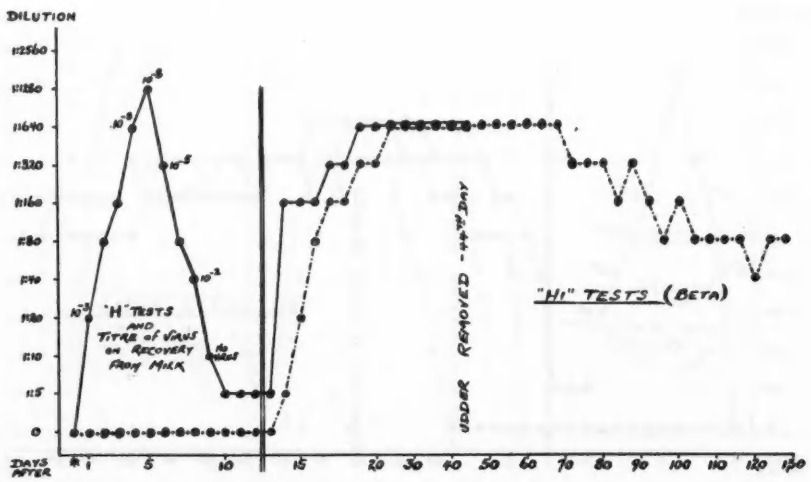

FIG. 6. Goat B22186, 2.0 cc. influenza "A" virus instilled in left half of udder.
* Day of inoculation.
———— Milk from inoculated (left) half of udder.
- - - - - - - Blood serum.

Results and Discussion

Reference to Figs. 1 and 2 indicate that shortly after the removal of either the whole gland or that half which had been the seat of propagation of Newcastle disease virus there was a precipitous drop in the antibody level of the blood. It is difficult to escape the conclusion that the gland was the principal seat of antibody production and that which was present in the blood was, in large measure, an overflow from the gland. The evidence, however, throws little light on the more basic background of whether antibody production is the result of a continuous stimulus or a function of tissue taken on following a short term stimulus. It has been established that once the virus disappeared, free virus could not be found either in the milk or samples of gland tissue which were removed surgically.

The instilling of influenza virus (Figs. 3, 4) brought about a somewhat different result. The blood titer fell following surgical removal of the gland or that part which propagated virus, but nevertheless there continued to be present in the blood for months a substantial level of antibody. It would, therefore, seem that either the production of antibody or the stimulus which brings about the production is not so intimately related to mammary gland tissue as in the case of Newcastle disease virus. This is emphasized by the result which followed the instillation of both viruses in the same host (Figs. 5, 6). Following the removal of the gland Newcastle disease antibody fell in two weeks to a low level whereas influenza antibody came down slowly and always continued to maintain a substantial level.

The trials which have been described together with those mentioned in a former paper (2) would appear to indicate that the mammary gland is the seat of a substantial production of neutralizing antibody when the virus has been instilled into the lactiferous sinus and propagation taken place. The question of whether a continuous stimulus is at work resulting in the formation of neutralizing antibody or whether a short term stimulus was capable of exciting the immune mechanism for long periods of time is left unanswered. It is difficult to visualize antibody production being maintained at a high level for months in the absence of a continuous stimulus. Some color is given to this view by the fact that following the instillation of both influenza and Newcastle disease viruses the antibody reaches a similar level but only in the influenza virus is this high level permanently maintained. The primary stimulus of each virus which called forth an antibody response does not appear to differ but since Newcastle disease antibody seems to appear and disappear it suggests that this stimulus is not long sustained and indeed that the falling off of antibody production may be related to the disappearance of continuous stimulus. That virus is not present in a free form does not rule out the possibility that it may be in a state which precludes recognition by available methods.

Acknowledgment

The assistance of Dr. Eric F. Pallister, who carried out the exacting surgery in this study, is acknowledged with grateful thanks.

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INFECTIOUS HEPATITIS. ATTEMPTS TO INFECT RODENTS AND TO ISOLATE VIRUS IN TISSUE CULTURES[1]

By A. E. Franklin, Barbara Buchner, Elizabeth McKee, J. C. Sinclair, W. G. C. Bearcroft, Nancy Oke, and C. E. van Rooyen

Abstract

The paper describes continued efforts to isolate the virus of infectious hepatitis. Experiments include a search for naturally-occurring hepatitis among wild rodents indigenous to Southern Ontario, captured in the proximity to the homes of human cases. Attempts have been made to infect similar animals with the faecal extracts and sera from human cases. Suckling mice were inoculated with infective material and tissue emulsions serially propagated for a period of over one year. Efforts to cultivate the virus in tissue culture of monkey liver, spleen, and lung are described in detail. A search for specific complement-fixing antibodies in convalescent sera and for haemagglutinins in inoculated tissue culture fluids has proved negative.

Introduction

Following experiments on human volunteers by MacCallum and Bradley (16) and Paul, Havens, Sabin, and Philip (24) it was demonstrated that infectious hepatitis was caused by an agent of the filterable virus category. Subsequently, repeated efforts were made to reproduce the disease in animals but without success. A group of 23 animals consisting of mammals and birds previously found to be refractory to infection with the virus of human infectious hepatitis has been reported by Morris, O'Connor, and Coburn (20).

The list of named genera and species are as follows:

Cat (*Felix domestica*)
Chipmunk (*Tamias striatus*)
Dog (*Canis familiaris*)
Ferret (*Mustela furo*)
Fox (*Vulpes fulva*)
Guinea pig
Swine
Mink (*Mustela vison*)
Mouse, Swiss, suckling
Mouse, A albino, subline L
Mouse, Swiss, 8–10 gm.
Mouse, gray (*Mus musculus*)
Mouse, white-footed (*Peromyscus leucopus*)
Opossum (*Didelphis virginiana*), suckling
Opossum (*Didelphis virginiana*), adult
Prairie dog (*Cynomys* sp.)
Raccoon (*Procyon lotor*)
Rat, albino
Rat, cotton (*Sigmodon hispidus*)
Rat, kangaroo (*Dipodomys* sp.)
Rat, pack (*Neotoma* sp.)
Rat, rice (*Oryzomys palustris*)
Squirrel, gray (*Sciurus carolinensis*)
Squirrel, ground (*Citellus* sp.)
Vole (*Microtus* sp.)
Woodchuck (*Marmota monax*)

Birds

Canary (*Serinus* sp.)
Duckling (*Anas moschata*)
Pigeon (*Columba* sp.)
Pheasant (*Phasianus colchicus*)
Quail (*Colinus virginianus*)
Starling (*Sturnus vulgaris*)

[1]*Manuscript received September 29, 1955.*
Contribution from the Connaught Medical Research Laboratories and the Department of Medicine, University of Toronto, Toronto, Ontario.

Determined efforts to infect rodents with blood and faeces from acute cases of the disease were conducted by MacCallum (15), Morris (19), and Mirick (17) but without effect. During the course of such studies naturally occurring enzootic hepatic diseases of mice have been observed at different laboratories and the existence of this is now recognized as a source of error, likely to confuse workers who attempt to propagate the virus of infectious hepatitis in mice (19). The disadvantages of the mouse as an experimental animal for hepatitis research is also stressed by Mirick (17) who distinguishes the prevalence of four types of mouse hepatitis virus, namely: Type I—described by Findlay (7), Thompson (29), Nicolau and Ruge (22), Olitsky and Casals (23), and Pavilanis and Lepine (25); Type II—infections encountered by Jordan and Mirick (13) and Lackey, Eichman, and Havens (14); Type III—mouse hepatitis originally described in 1951 by Gledhill, Dick, and Andrewes (8) representing a mixed type of infection caused by a viral agent acting in conjunction with a protozoan parasite, *Eperythrozoon coccoides*, which is susceptible to the action of terramycin (see also (4)); Type IV—a hepatitis virus associated with the presence of anaemia in suckling mice investigated by Nelson (21).

Earlier, unsuccessful efforts to transmit the agent of infectious hepatitis to swine were recorded by van Rooyen and Gordon (30). Further experiments on swine were conducted by Syverton, Young, and Brunner (28) in 1954 who employed 16 miniature antibody-free newborn swine, but their efforts were also a failure. Primates other than man have likewise been tested for susceptibility. Evans (6) has reviewed the literature relating to the use of these animals, but no evidence was obtained to suggest that primates including chimpanzees were susceptible to infection. Simultaneously with efforts to transmit the agent of human infectious hepatitis to laboratory animals, attention has been directed towards viruses causing hepatitis in certain species of animals, in the hope that comparative studies might shed some light on the aetiology of human infective hepatitis. Gochenour (9) has reviewed the literature dealing with certain virus diseases resembling infectious hepatitis such as: equine influenza, virus abortion disease, infectious canine hepatitis or enzootic encephalitis, canine distemper, hard pad disease of dogs, feline panleucopenia, hog cholera, and raccoon jaundice. Indirect approaches such as these have likewise failed to solve the riddle of human infectious hepatitis. Chicken embryos have been extensively employed as a medium for the attempted propagation of this elusive virus. The results, however, have been both confusing and inconclusive and research on this phase of the problem has been summarized by Henle (12) and Dalldorf (3).

Comparatively recently, a claim has been advanced by Pollard and Diserens (26) who reported that the injection of acute serum from cases of human infectious hepatitis in the allantoic sac of seven-day-old embryonated eggs resulted in a marked increase in the histiocyte cell content of that fluid. Pollard and Diserens (26) asserted that the effect could be inhibited by convalescent

human infectious hepatitis serum and that it could not be produced by the injection of serum from cases of simple obstructive jaundice.

Developments in the field of poliomyelitis research following the introduction of tissue culture techniques by Enders, Weller, and Robbins (5) have focussed attention on the possibilities offered by the application of tissue culture methods for the isolation of infectious hepatitis virus. Human tumor cell lines including the HeLa strain, derived from a case of carcinoma affecting the cervix, have also been employed (2, 27, 11). In our experiments a range of human cells has been tested for susceptibility to the virus of infectious and serum hepatitis but without encouragement. Human embryonic liver, muscle, gut, and kidney tissue have been set up in roller tube cultures, inoculated with serum from acute cases of infectious hepatitis and examined for evidence of destructive effect but so far without success. A full account of these tests will be reported in a subsequent publication.

The failure of infectious hepatitis virus to show cytopathogenic effect in a variety of human tissue cultures is disappointing. It should be remembered, however, that numerous other methods of cultivation remain to be explored and many normal, embryonic, and abnormal animal and human varieties of cells, including pure lines of cells and various mutant strains, have not yet been examined for evidence of cytopathogenic effect. Likewise the susceptibility of cells tested under varying conditions of growth remains to be investigated (1).

At the present time, it may be said that the viruses of infectious hepatitis and serum jaundice have stubbornly refused to reveal demonstrable evidence of cultivation by conventional techniques. A search for specific serological reactions has also proved to be a failure according to Havens (10). The future assault on this problem should therefore be directed towards a systematic step-by-step elimination of each likely method of cultivation. Under the circumstances, the publication of negative results acquires special significance as data for the guidance of successive investigators.

Attempts to Infect Suckling Mice with Infective Human Material

In view of the susceptibility of mice to a wide range of virus infections we have repeated and extended earlier efforts to infect these animals by the use of two-day-old suckling mice.

Our tests have been performed with faeces, plasma, and serum gathered from patients suffering from infectious hepatitis contracted in the province of Ontario. The latter included Metropolitan and Suburban Toronto, Toronto Centre Island, Peterboro, Lakefield, and the surrounding districts. To our knowledge, strains of infectious hepatitis virus derived from patients in Ontario have not been examined for mouse pathogenicity.

Altogether stools from 26 patients, and blood from six of these, were employed in the present studies. Faeces were collected at the patient's home, placed in dry ice, and transported to the laboratory by car. Simultaneously a specimen of blood was collected and transported to the laboratory with minimum delay.

Preparation of Faecal Extracts

An approximate 1 : 10 dilution of faecal material was prepared in nutrient broth and 1000 units of penicillin and streptomycin were added. The mixture was next filtered through a Whatman No. 1 filter, passed through a Seitz EK grade filter, and used for inoculation. A few specimens were treated differently by preliminary extraction with ether in order to remove excess fatty content. Certain other specimens were freed from bacteria by the addition of antibiotics and differential high speed centrifugation as a substitute for filtration.

Inoculdition of Mice

Suckling two-day-old mice were inoculated intracerebrally with 0.01 ml. of material as prepared above. Mice of this age appeared to withstand such inoculation and only a few non-specific deaths occurred. Likewise, mice of the same age were inoculated intraperitoneally with 0.05 ml. of inoculum. After 14 days, mice were killed, their brains removed, a 1 : 10 emulsion of same prepared in nutrient broth, antibiotics added, and further animals inoculated. In this manner each specimen derived from 23 different patients was inoculated in an average family of eight to nine mice and carried for six transfers over intervals of 14 days between each passage. When the sixth passage was reached, namely the 84th day after the original inoculation, the mice were killed, the brains removed, placed in fixative, histological specimens were prepared, stained, and duly examined.

In the case of mice inoculated intraperitoneally, the liver and spleen of each animal was pooled and passaged as above. Histological specimens of liver were prepared at each passage. More than 1000 histological sections of liver were prepared, stained with haematoxylin and eosin, and examined for viral lesions but none were found. In a single specimen we noticed some degree of focal and zonal damage to liver parenchyma with varying amounts of periportal cellular infiltration. The lesions were however, neither constant in character nor regularly reproducible on subsequent passage. It was thus concluded that these lesions might have been due to spontaneous mouse hepatitis as already encountered by other workers.

Since the mechanism of adaptation of certain viruses to new hosts is well known to be a slow process, we continued to make transfers for a total period of one year. Thus three pools of liver and spleen were prepared from the sixth intraperitoneal passage and carried for 16 further passages at two-week intervals over a period of 12 months, but again with negative results. Simultaneously, a duplicate set of mouse passages was conducted with pooled faecal material at four week intervals for 11 passages over 12 months, but again with negative results.

A Search for Naturally Occurring Hepatitis in Wild Rodents Captured in Proximity to Human Cases

So far as is known, there has been no evidence to indicate that human hepatitis is in any way related to naturally occurring hepatitis in laboratory mice or to any other rodent either wild or bred in captivity. In Ontario, we

have noted that small and localized outbreaks of infectious hepatitis have frequently occurred in relatively isolated rural communities. Amid such surroundings the first cases appear to have arisen spontaneously without any obvious history of person to person contact. Likewise, the mechanism by which cases are carried from one winter to another in an endemic area has interested us. The frequent association of cases with bad domestic sanitation and poor personal hygiene has been observed by ourselves and others. Amid these surroundings we have deemed it advisable to examine wild rodents as possible factors in the dissemination of infection. Thus rodents indigenous to Southern Ontario were captured in close proximity to human cases and examined for histological evidence of hepatitis. Furthermore we have tested the susceptibility of wild rodents (together with a small number of clean animals which we were able to breed in the laboratory) by inoculation with human faeces from cases of hepatitis.

At one home where six cases had occurred at Richvale, Ontario, six rodents of the species *Peromyscus maniculatus bairdii* were trapped on the grounds. Likewise 19 other rodents of the same species were gathered near other infected homes. Control animals were captured at a distant site, named Long Point on Lake Erie, which is remote from human habitation. Histological sections were prepared from these animals and in a single mouse captured at Long Point, Ontario, we observed some degree of liver damage of uncertain aetiology. The remaining animals captured near the homes of human cases of infective hepatitis showed no evidence of liver damage.

Attempts to Infect Other Wild Rodents

The following new species were tested for susceptibility but with negative results. These names should therefore be added to those already tested and found to be refractory: *Peromyscus maniculatus bairdii* (prairie deer mouse), *Peromyscus leucopus novoboracensis* (prairie deer mouse), *Peromyscus maniculatus gracilis* (prairie deer mouse), *Tamiscurus hudsonicus* (red squirrel), *Microtus pennsylvanicus* (vole or meadow mouse), *Mus musculus* (house mouse), and the Canadian chipmunk.

The above animals were inoculated intraperitoneally with 0.25 to 0.50 ml. of faecal extract prepared as described above. They were observed for a period of four to six weeks, but no signs of illness were detected. Histological sections were prepared from 87 animals but no significant evidence of damage could be produced with regularity with any given sample of infective material.

Tissue Cultivation Experiments

For purposes of record and assistance to others who may wish to repeat our work, a detailed description of our methods is provided. Monkey liver, spleen, and lung cultures were employed for our tests.

Preparation of Glassware

All glassware was washed in concentrated sulphuric acid containing 0.5% sodium nitrite and 0.5% sodium hypochlorite, followed by repeated rinsings in hot tap water, distilled water, and finally in double-distilled water. It was

sterilized by autoclaving or by dry heat. White rubber stoppers were boiled in a 1% Alconox solution followed by similar rinsings and were sterilized by autoclaving.

Preparation of Cultures

Monkey tissues were obtained as aseptically as possible at operation from butylone-anaesthetized rhesus and cynomolgous monkeys. The excised tissues were placed immediately in a nutrient medium containing penicillin and streptomycin and transported without delay to the laboratory.

The tissues were placed in separate sterile Petri dishes, and connective tissues, fatty material, and blood clots were discarded. In the case of liver, the gall bladder was excised. Each tissue was then placed in a sterile container and washed with three successive changes of approximately 30 ml. of nutrient medium which contained 500 units per ml. of penicillin and 500 μgm. per ml. of streptomycin. The tissue was then minced until the fragments were 1 to 2 mm. in diameter and again washed until the supernatant was relatively clear. The washed mince was left to stand at room temperature for about one hour before being set up in tissue culture.

For stationary cultures, two or three drops of the prepared tissue culture suspension were added to 25 ml. Erlenmeyer flasks, using a large bore Pasteur pipette. Care was taken to ensure that the same amount of tissue was present in each culture. Three milliliters of nutrient medium were added to each culture and the flask was stoppered tightly. In the case of rotating cultures, plasma-clotted 18 mm. × 150 mm. roller tube cultures were prepared as follows: Five milliliters of reconstituting fluid were added to one vial of "Difco" chicken plasma and shaken until solution was complete. All materials used in connection with this solution were kept either in an ice bath or in a cold room at 5° C. Two milliliters of reconstituting fluid and 5 ml. of synthetic medium were added to one vial of "Difco" EE 100 chicken embryo extract and shaken until solution was complete. The extract was then centrifuged for about five minutes at 1500 r.p.m. and the supernatant removed. This solution was not kept refrigerated.

Roller tubes were thoroughly chilled before using. One drop of chilled chicken plasma was added from a chilled syringe. The plasma was ringed around the lower third of the tube, using a sealed Pasteur pipette. Ten to twelve tissue fragments were then placed in a straight line in the tube. After approximately fifty cultures had been prepared in this manner, one drop of chick embryo extract was run over the line of fragments. Roller tubes were left at room temperature for about thirty minutes before 2 ml. of nutrient medium were added. All cultures were tightly sealed with white rubber stoppers and placed in a rotating machine at 1/5 r.p.m. in the incubator.

The most common nutrient media used in these studies were synthetic mixtures No. 199, 635, and 697 as devised by Morgan, Morton, and Parker (18). In most cases these media were supplemented by the addition of horse serum, ranging in concentrations from 1% to 10%. These media were prepared in our own laboratories and were sterilized by Seitz filtration.

All cultures were maintained at 37° C. during the course of these experiments, and were not gassed since pH estimations were made prior to replacement of medium. The supernatant fluids were removed, and replaced with fresh medium twice weekly or when the pH of the fluids decreased to pH 6.8. Cultures were maintained as long as the culture was metabolizing or as long as the explant appeared to be in a healthy condition. The pH of individual cultures was estimated, to the nearest 0.1 pH unit by comparison with known standards, immediately prior to replacement of the nutrient medium, and the average pH of a group of cultures was made mathematically. Furthermore, a comparison was made between the pooled control and pooled infected fluid.

The number of cultures used during this study has varied from three to fifty cultures for investigation of each inoculum and an equal number of controls were included. Erlenmeyer cultures, as a rule, were infected 24 hr. after preparation of the cultures, but sometimes as late as 16 days after preparation. Some roller tube cultures were infected on the day of preparation, others were infected at periodic intervals, during the subsequent outgrowth of the original explant.

Preparation of Virus Inoculum

Acute stage infectious hepatitis bloods were collected as soon as possible after diagnosis had been made, usually within a few days after the onset of jaundice, and placed in a heparinized roller tube. On receipt in the virus laboratory, the blood sample was centrifuged, the plasma removed, and stored in a deep-freeze chest until required for experimental purposes. Acute stage stool specimens were deep-frozen until an extract was made. A 10% suspension of stool in sterile saline was centrifuged for 30 min. at 2500 r.p.m. Five hundred units per milliliter of penicillin and 500 μgm. per milliliter of streptomycin were next added to the faecal supernatant, which was then distributed in 5 ml. aliquots and stored in the deep-frozen chest. In all instances, 0.2 ml. of infected serum was added to each culture. When possible, a single sample was employed. However, it was sometimes necessary to pool several plasmas to provide sufficient inoculum. Faecal extracts have not been pooled and 0.2 ml. of undiluted faecal extract was added to Erlenmeyer cultures and 0.1 ml. was added to roller tube cultures.

Subcultivation of Virus-suspect Inocula

Virus-suspect specimens which had been stored in the deep-freeze were subcultivated by infecting a freshly prepared culture with 0.2 ml. of a fluid change representing 10 to 30 days' incubation after addition of the original culture of virus-suspect specimen.

Results

A summary of the experiments which have been carried out is presented in Tables I, II, and III. In Table I it will be seen that a total of 125 experiments have been carried out in cultures of monkey spleen. Eleven different acute stage plasmas and 11 different acute stage faecal extracts were used. A

TABLE I

SUMMARY OF A NUMBER OF EXPERIMENTS ON CULTURES OF MONKEY SPLEEN INFECTED WITH ACUTE STAGE PLASMA AND FAECAL EXTRACTS OF PATIENTS WITH INFECTIOUS HEPATITIS

	Erlenmeyer cultures		Roller tube cultures	
	Plasmas	Faecal extracts	Plasmas	Faecal extracts
Original cultures	14	36	24	1
Primary subcultures	8	8	7	3
Second subcultures	3	9	8	—
Third subcultures	1	1	1	—
Fifth subcultures	—	—	—	1
Total	26	54	40	5

TABLE II

SUMMARY OF A NUMBER OF EXPERIMENTS ON CULTURES OF MONKEY LIVER INFECTED WITH ACUTE STAGE PLASMA AND FAECAL EXTRACTS OF PATIENTS WITH INFECTIOUS HEPATITIS

	Erlenmeyer cultures		Roller tube cultures	
	Plasma	Faecal extracts	Plasma	Faecal extracts
Original cultures	3	8	11	2
Primary subcultures	—	2	1	2
Second subcultures	—	—	1	—
Total	3	10	13	4

TABLE III

SUMMARY OF A NUMBER OF EXPERIMENTS ON CULTURES OF MONKEY LUNG INFECTED WITH ACUTE STAGE PLASMAS AND FAECAL EXTRACTS OF PATIENTS WITH INFECTIOUS HEPATITIS

	Erlenmeyer cultures		Roller tube cultures	
	Plasmas	Faecal extracts	Plasmas	Faecal extracts
Original cultures	—	4	6	—
Total	—	4	6	—

number of subcultures have been attempted also. In Table II it will be seen that a total of 30 experiments have been carried out in cultures of monkey liver. A number of primary subcultures have also been attempted. Similarly in Table III it will be seen that a total of 10 experiments has been carried out in cultures of monkey lung using one acute stage plasma and two acute stage faecal extracts. No subcultures of virus in this tissue were attempted.

During the course of these experiments it was observed that there was no evidence of a cytopathogenic effect produced by the acute stage plasmas or acute stage faecal extracts which had been used as inocula. However, during

some of the experiments we noted that the pH of the pooled infected supernatant fluids was frequently lower than the pH of the corresponding pooled control supernatant fluids. It was considered that this effect might have been due to virus action and as such, would be transmissible to another culture. Accordingly, a number of subcultures was carried out using supernatant fluids in which this phenomenon was noticed. A large number of experiments was performed to explore this possibility but unfortunately many of the fluids could not be successfully subcultured and many of the experiments were inconclusive. A statistical evaluation of our results did not indicate that the phenomenon noted was significant.

Serological Studies

Fluids were harvested from monkey cell tissue cultures previously inoculated with infected material and were used for testing for evidence of haemagglutinins and for complement-fixing antibodies that might be specific for infective hepatitis virus.

Haemagglutination tests were performed in plastic plates or test tubes employing an initial 1:2 dilution of tissue culture fluid to which an equal volume of an erythrocyte suspension was added. Conditions were varied to include the following features: Two concentrations of saline, namely, 0.85 and 1.70%; three different temperatures of incubation, 37° C., 20° C., and 4° C. Erythrocytes from human (type O), sheep, chicken, and the rabbit were employed in concentrations of 1% for human and sheep cells and 0.5% and 1.5% for chicken and rabbit cells. More than 70 tissue culture fluids, ranging from the first to the seventh fluid change, were examined for evidence of haemagglutinins but none were found.

Complement fixation tests were done using undiluted culture fluids as antigens and doubling dilutions of convalescent serum from infectious hepatitis patients. Two M.H.D. of guinea-pig complement and a 2% suspension of sheep red cells were used in the tests. The mixtures of serum and antigen were incubated at 37° C. for one and one-half hours prior to addition of the haemolytic system. Fluids from inoculated tissue cultures of both monkey and human origin were included in the tests. No evidence of specific complement-fixing antibodies were found.

Conclusions

Attempts to propagate the virus of infectious hepatitis in tissue cultures of monkey spleen, liver, and lung, supplied with synthetic nutrient mixtures and horse serum, have indicated that this agent did not exhibit an obvious cytopathogenic effect under these conditions. A total of 80 Erlenmeyer and 45 roller tube cultures of monkey spleen were inoculated with infective material. In 30 experiments monkey liver tissue was used for which six acute stage plasmas and four acute stage faecal extracts were employed as infecting material. Ten experiments were conducted using monkey lung tissue cultures.

and these were inoculated with one acute stage plasma and two acute stage faecal extracts. The pH values of pooled supernatant fluids were recorded in all experiments.

Attempts to isolate the virus of infective hepatitis by other methods are described in detail under their respective headings and summarized in the abstract.

Efforts to infect wild rodents indigenous to Southern Ontario as well as suckling white mice have been unsuccessful. Likewise a search for specific haemagglutinins and for complement-fixing antibodies using tissue culture fluids as antigen has proved to be negative.

Acknowledgments

This work has been undertaken with the aid of Defence Research Board Grant number 8915-04 for the study of the aetiology of infectious hepatitis.

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ABNORMAL MORPHOLOGY OF A BACTERIUM RESULTING FROM VITAMIN B_{12} DEFICIENCY[1]

By C. E. Chaplin and A. G. Lochhead

Abstract

Studies of a vitamin B_{12}-requiring bacterium of the genus *Arthrobacter* showed that with suboptimal concentrations of the vitamin growth in liquid medium was flocculent whereas with adequate amounts of B_{12} cultures were uniformly turbid. Flocculation, which was progressively more pronounced with decreasing vitamin supply, was associated with abnormal cell morphology. Cells were noticeably swollen and elongated, irregularly bent, and showed rudimentary branching. The critical concentration of vitamin B_{12} below which morphological changes began to occur was approximately 0.3 mμgm. per ml.

Introduction

In a study of the response of various vitamin B_{12}-requiring bacteria to different concentrations of the vitamin it was noted that whereas in the presence of adequate amounts of the vitamin liquid cultures showed uniform turbidity, certain strains were found to show flocculent growth with the lower levels of B_{12}. The present report deals with morphological changes found to be associated with the flocculation which resulted from a deficiency of the vitamin.

The organism selected, No. 38, was one of a group of vitamin B_{12}-requiring strains isolated from soil by Lochhead and Thexton (2). Of these, 19 strains were described in detail by Lochhead and Burton (1). On the basis of morphological and physiological tests in which appropriate media were employed with the incorporation of soil extract or vitamin B_{12} at a concentration of 2 mμgm. per ml., culture No. 38 was regarded as a species of *Arthrobacter* and found to require, in addition to vitamin B_{12}, biotin and thiamine (1).

Experimental

For studies on the effect of vitamin B_{12} concentration, the basal medium used was one containing yeast extract (Difco), inorganic salts, and glucose. This medium, previously described as Medium Y (1, 2) contained an adequate supply of biotin and thiamine. For the tests it was modified by the addition of crystalline B_{12} to provide a suitable range of concentrations of the vitamin, 10 ml. quantities being dispensed in 50 ml. Erlenmeyer flasks. Following sterilization the flasks were inoculated with one drop of a washed suspension of a three-day stationary culture of No. 38 in 10 ml. of basal medium containing 2 mμgm./ml. of B_{12}. The flasks were incubated for 48 hr. in a shaker at 26° C.

[1]*Manuscript received November 21, 1955.*
Contribution No. 398 from Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa.

Results

At the end of the period of incubation the flasks were shaken vigorously by hand to break up and distribute the clumps for turbidimetric estimation of the growth. A typical growth–response curve is shown in Fig. 1 where, it will be seen, maximum density is approached at about 0.3 mμgm. B_{12}/ml.

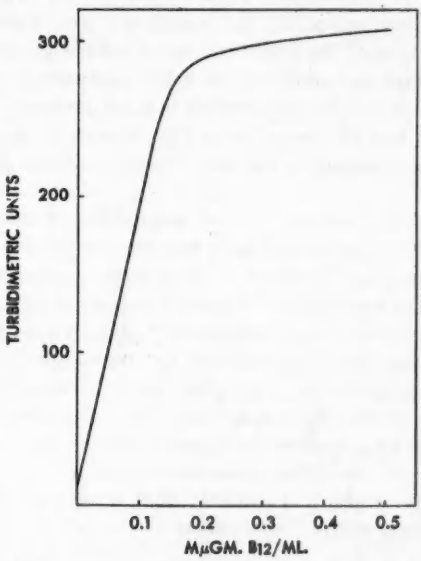

FIG. 1. Growth–response curve of Culture No. 38 in Medium Y in relation to vitamin B_{12} concentration.

In a parallel series of flasks cultures were obtained for morphological examination. Films were Gram-stained and photographed. Growth in concentrations of B_{12} adequate for normal development of the organism is shown in Figs. 2 and 3, where the visible difference is in size only; the higher concentration (Fig. 2; 2 mμgm./ml.) producing somewhat smaller cells than the lower (Fig. 3; 1 mμgm./ml.). Slight clumping is seen to commence at 0.3 mμgm./ml. (Fig. 4) associated with the presence of somewhat swollen and bent cells. These conditions are further developed at 0.2 and 0.15 mμgm./ml. (Figs. 5 and 6) where Gram-negative parts of the cell begin to appear. At lower levels the abnormalities are still more pronounced where rudimentary branching can also be seen (Fig. 7: 0.075 and Fig. 8: 0.05 mμgm./ml.). Below this level free cells become increasingly rare, the bulk of the growth being contained in the relatively few large flakes which characterize growth in the suboptimal concentrations of vitamin B_{12}. The control flask to which no B_{12} was added showed some very slight flaky growth and only an occasional abnormal free cell (Fig. 9). Part of a flake obtained from a culture at 0.1 mμgm. is shown in Fig. 10. These flakes exist in all cultures up to 0.3 mμgm. Below a B_{12} concentration of 0.075 mμgm./ml. the supernatant is clear;

thereafter it becomes increasingly turbid to a near maximum at 0.3 mμgm./ml. while the flakes disappear close to this level. When flakes were transferred to B_{12}-adequate medium normal cells resulted.

Discussion

In the lower concentrations of B_{12} the turbidity curve is steep and it could be suggested that because of the flocculent nature of the growth and the clear supernatant the readings obtained are not truly comparable with those from the higher concentrations where the growth is homogeneous. This would be a valid objection if it had not been found that strains of other B_{12}-requiring organisms showing no clumping in the lower concentrations gave very similar turbidity curves.

The reason for the flocculation is not apparent. It does not resemble agglutination in that it is associated with the growth process and not with a reaction subsequent to growth; there is no apparent adhesive material such as a polysaccharide; an electrolytic or surface active additive does not cause dispersion. The aberrations in cell morphology are perhaps of greater interest and in a general way may be compared with the forms obtained from cultures in adverse conditions of antiseptic, antibiotic, or salt concentrations, but the present case is unique in that the cause is a vitamin deficiency. Confirmatory evidence was obtained by transferring abnormal cells to a B_{12}-adequate solid medium where the whole population was observed to revert to normal growth habit and morphology, making it unlikely that a vitamin B_{12}-independent, morphologically aberrant mutant was selected.

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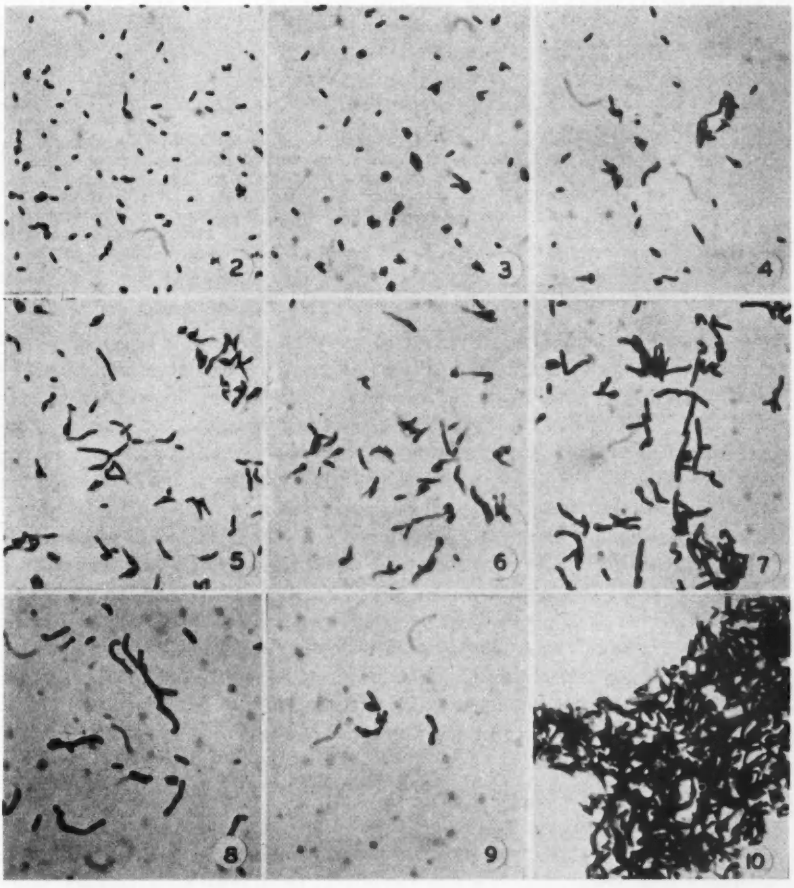

FIGS. 2 to 10. Growth at various concentrations of vitamin B_{12}. Gram-stain. × 1100. FIG. 2. 2 mμgm./ml. Normal cells. FIG. 3. 1 mμgm./ml. Normal cells, slightly larger. FIG. 4. 0.3 mμgm./ml. Slight clumping; some irregular cells appearing. FIGS. 5 and 6. FIG. 5, 0.2 mμgm./ml.; and FIG. 6, 0.015 mμgm./ml. Irregular, elongated, and bent cells predominating. Gram-negative sections of cells visible as gray. FIG. 7. 0.075 mμgm./ml. Cells swollen and much elongated with slight branching. FIG. 8. 0.05 mμgm./ml. More pronounced branching; large Gram-negative areas. FIG. 9. 0 mμgm./ml. Very few cells, distorted. FIG. 10. 0.1 mμgm./ml. Section of flake. Irregular Gram-staining.

INFECTION DUE TO SALMONELLA PARATYPHI A

CASE REPORT[1]

By Philip Greey

Abstract

A case of infection due to *Salmonella paratyphi* A in an Italian housewife, who had been resident in Canada for two years, is presented. On two occasions the microorganism was cultured both from the blood stream and from faeces. Infection due to *S. paratyphi* A in Ontario is extremely rare. In the case described it is suggested that the infection was acquired from a friend who had recently arrived from Italy and who had suffered from diarrhea whilst staying in the patient's house. This, however, was not established bacteriologically.

Introduction

The isolation of *S. paratyphi* A from clinical material is extremely uncommon in Canada. Foley *et al.* (3) described the investigation of an outbreak in a small general hospital in Montreal which occurred in 1935 and stated, "this is the first time the organism has been reported found in Canada". In 1937 Ferguson and MacNabb (2) reported the isolation of *S. paratyphi* A from two blood cultures from a patient with paratyphoid fever. This was the first time the microorganism had been isolated at the Central Laboratory of the Ontario Department of Health. Since then *S. paratyphi* A has been cultured at the Central Laboratory on three or four occasions (1). Professor E. G. D. Murray (4) still possesses a culture of the strain of *S. paratyphi* A isolated in Montreal in 1935 and may have identified the culture. Since Dr. Murray was associated with the first isolation of *S. paratyphi* A in Canada it is perhaps appropriate to record in this issue of the Journal in his honor, another instance of infection due to the microorganism, the first encountered in the bacteriological laboratory of the Toronto General Hospital.

Case History

The patient was a 33 year old Italian housewife who had been living in Canada for the past two years with her husband and two children. She had not been away from Toronto in recent months. She was apparently perfectly well until May 15, when following dinner she had a chill lasting 10 to 15 min. followed by a feeling of feverishness. She also complained of headache and had sore eyes. She felt quite well for the next two days. On May 18 she had a recurrence of the chill accompanied by headache, sore eyes, and a feeling of feverishness. These same symptoms occurred for the next four days preceding her admission to the Toronto General Hospital on May 23. For two days prior to admission her appetite had been very poor. At the onset

[1]*Manuscript received October 27, 1955.*
Contribution from the Department of Bacteriology, University of Toronto, and the Toronto General Hospital, Toronto, Ontario.

of her illness she had been seen by her physician who had given penicillin for two days. There had been no preceding cold, sore throat, gastrointestinal symptoms, or chest pain.

On admission the patient appeared seriously ill. Temperature was 104.6° per rectum. Physical examination of the head, neck, and chest was negative. The heart was not enlarged and no murmurs were heard. Heart rate was 85 per min. Blood pressure 115/75. The liver and spleen were not enlarged. There was no diarrhea, abdominal tenderness, and no masses were felt.

Laboratory Examination

Hb. 12.9 gm., white count 4500 with 62% neutrophiles. Urine had a specific gravity of 1005 with a trace of albumin. Wassermann, tuberculin, Q fever, and cold agglutinin tests were negative.

Blood culture done on May 23 grew *S. paratyphi* A. Twenty-two colonies grew in a poured culture plate containing 1 ml. of the patient's citrated blood. Blood culture on May 26 also grew *S. paratyphi* A but colonies did not develop in the poured plate containing 1 ml. of the patient's blood. Three subsequent blood cultures gave no growth. The strain of *S. paratyphi* A grown from the blood was found, by a broth dilution technique, to be sensitive to less than 5 micrograms per milliliter of streptomycin and chloramphenicol. It was also sensitive to 2 micrograms per milliliter of tetracycline.

Of 11 stool cultures performed during the patient's stay in hospital, two yielded *S. paratyphi* A. Repeated cultures of urine gave no growth.

On May 24 the Widal agglutination test was negative but by May 29 agglutination occurred in dilutions of the patient's serum up to and including 1 : 320 both with a standard strain of *S. paratyphi* A and with the strain isolated from her.

Progress

Following admission the patient's temperature was continually elevated, varying between 100° and 102.8° F. Treatment with chloramphenicol was started on May 28, 3 gm. being administered daily, 500 mgm. q.4h. × 6. By May 30 her temperature was normal and remained so throughout her stay in hospital except for a slight rise on June 1. The patient's symptoms also rapidly subsided. Treatment with 3 gm. of chloramphenicol was continued until June 8 when the dose was reduced to 2 gm. daily. The patient was discharged on this day to continue on therapy for another 10 days making a total of three weeks of treatment.

Discussion

The source of infection in this case was obscure until it was learned that a friend, newly arrived from Italy five months before, had lived with the family until one week before the patient's illness. He had suffered diarrhea during the month of January of sufficient severity to cause him to see a doctor.

Efforts to trace this individual and obtain specimens for culture were not successful. It is highly likely, however, that he was the source of infection with *S. paratyphi* A which he had acquired in Italy and brought to Canada.

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PHAGE-TYPING AND ANTIBIOTIC-RESISTANCE OF STAPHYLOCOCCI ISOLATED IN A GENERAL HOSPITAL[1]

By E. T. Bynoe, R. H. Elder, and R. D. Comtois

Abstract

Staphylococcus aureus isolated from various sources in a hospital, from March, 1953, to March, 1954, were phage-typed and tested for sensitivity to seven antibiotics. Of the dressings from 1400 clean operations, 15.7% were found to contain *S. aureus* and 8% of the postoperative wounds were clinically infected. Of 516 cultures, 95.4% were typable with the 32 phages used. Group III strains were predominant in the cultures from the patients and showed a higher degree of resistance to many of the antibiotics than did the strains of Groups I, II, and IV or the strains isolated from student nurses on starting their training, before exposure to the hospital environment. While a high proportion of strains in the hospital were resistant to the more commonly used antibiotics, penicillin, chlortetracycline, oxytetracycline, and streptomycin, very few strains were resistant to erythromycin, chloramphenicol, or neomycin. A new phage type '81', the causative agent of 50% of the boils and abscesses investigated, was discovered. This has been found to be a common strain in Canadian hospitals and its similarity to the Australian hospital strain '80' is pointed out.

Introduction

Numerous reports from many parts of the world have stressed the recent increase of staphylococcal infections in the hospital. Concurrently, many of the staphylococci isolated during these studies were found to be resistant to the antibiotics, penicillin and the tetracyclines, in common hospital use.

An apparent increase in the number of staphylococcal infections, particularly of surgical wounds, occurring in one of our local hospitals prompted a study of this problem. The development of the bacteriophage typing of staphylococci by Fisk (4), Wilson and Atkinson (21), and Williams and Rippon (19) has provided a means of identifying strains of *Staphylococcus aureus* (*Micrococcus pyogenes*) and has proved a most useful epidemiological tool. The importance of phage group III staphylococci in "hospital infections" has been pointed out by most of the investigators who have studied these "hospital" staphylococci (5, 10, 14, 16, 20). Moreover, in many of these investigations, attention has been drawn to the association of penicillin-resistance of the strains with lysis by group III bacteriophages. It seemed that the logical approach to an investigation of staphylococcal infections in a hospital would be first to identify as specifically as possible the staphylococci involved. Phage-typing offered the best means available of strain identification and it is the purpose of this paper to report the typing of 516 cultures of *S. aureus* isolated from an Ottawa hospital, from March, 1953, to March, 1954, the resistance of these strains to seven antibiotics and their relationship to the environment (infections, carriers, etc.).

[1]*Manuscript received October 15, 1955.*
Contribution from the Laboratory of Hygiene, Department of National Health and Welfare, Ottawa, Ontario.
This paper was presented in part at the meeting of the Laboratory Section, Canadian Public Health Association, held in Montreal, Quebec, December, 1954.

Methods

Source of Cultures

In order to gain some information on the distribution of coagulase-positive staphylococci in the hospital, the first dressing removed after all surgical operations (except colostomies and the like, in which dressings would obviously be heavily contaminated) were routinely examined for the presence of *S. aureus*. Dressings or swabs from all suspected staphylococcal infections, such as boils, carbuncles, etc., were also included in the study. Nose and throat swabs from 95 student nurses on starting their training (before exposure to the hospital environment), from the same group three months later, and from a number of nurses who had been on the wards for some time were also cultured for pathogenic staphylococci. A number of plates were exposed to the air of one of the wards (the pediatric) and examined for *S. aureus*. All dressings and swabs were plated on 5% human-blood agar, and in some instances on Bacto-Chapman Stone Medium as well, and "suspect" colonies of *S. aureus* were transferred to nutrient agar slopes. These were examined morphologically and tested for coagulase by both tube and slide methods (6, 2) using human plasma. All Gram-positive coagulase-positive staphylococcus cultures were then phage-typed and tested for their sensitivity to antibiotics.

Phage-typing

The method of phage-typing was that recommended by Williams and Rippon (19) with a few minor modifications. The medium used for typing and for propagation of the phages was B.B.L. trypticase soy agar and phages of satisfactory titer were filtered through Zsigmondy membrane filters (22), which were found to cause less loss of titer than did Seitz filters. The strains for typing were grown in B.B.L. trypticase soy broth and incubated at 37° C. for 18 hr. All test plates for typing were incubated at 37° C. for five hours followed by holding at room temperature overnight. The phages and propagating strains were the same as those used by Williams and co-workers at the International Staphylococcus Phage-typing Reference Centre at Colindale, England. At the start of the study, 22 phages (3A, 3B, 3C, 6, 7, 29, 29A, 31, 42B, 42C, 42D, 42E, 44, 44A, 47, 47A, 47B, 47C, 51, 52, 52A, and 57) were used routinely. Later in the study, nine additional phages (53, 54, 55, 70, 73, 75, 76, 77, and 79) were received from Dr. R. E. O. Williams and were included in the routine testing of the cultures. All cultures which had previously been found insensitive to the original 22 phages were tested against these nine additional phages. Despite the use of 31 phages for typing, a large number of strains were still untypable and an attempt was made to find a phage or phages which would lyse them. One such phage was successfully developed by adaptation of phage 42B on one of these strains (L.H. 66459). This phage was found to attack a large number of these previously untypable cultures, and together with its propagating strain was sent to Dr. Williams at Colindale, who confirmed our findings and labelled it phage number '81'. This phage

was added to the other 31 routine test phages, and all cultures not previously checked against this phage were retested. The routine procedure was to test all cultures first against the 32 phages at their routine test dilutions (R.T.D.) and, if any lysis was observed, to record the results and to do no further typing tests. If no lysis was observed, the test was repeated using all 32 phages diluted 1:10 in order to eliminate some of the false reactions which occur with undiluted phage. According to Wahl and Fouace (17) and to Williams and Rippon (19), undiluted phage may often give clear zones resembling true lysis which are due to some agent, not yet identified, which inhibits the growth of the staphylococci. If no lysis was obtained with the 1:10 dilutions of the phages, the strain was considered 'not typable'. If lysis occurred with the 1:10 dilutions, the phages which caused this lysis were then titrated against the culture using 10-fold dilutions of phage, in order to determine the maximum dilution at which lysis occurred. Only the results obtained at the maximum test dilution, viz., 10 × R.T.D., 100 × R.T.D., were recorded. To illustrate with a specific example, culture A is tested against all the phages at R.T.D. No lysis is observed. The culture is retested against all phages diluted 1:10. Lysis is now observed with phages 42B, 47C, and 52. The strain is again retested with these three phages at R.T.D., 10 × R.T.D., 100 × R.T.D., up to a 1:10 dilution of the concentrated phages. Lysis occurred with the three phages at 100 × R.T.D. but only with phage 52 at 10 × R.T.D. Only that lysis caused by phage 52 is considered in reporting the results. In recording results, the recommendations of Williams and Rippon (19) were followed, and strains were identified according to their "pattern of lysis". Only the strong lytic reactions, that is, more than 50 plaques, were taken into account in recording the pattern. As pointed out by these authors, and as most workers in this field have found, most of the phages used "appear to give pattern reactions rather than truly type-specific lysis". The more phages used in the typing, and the more thorough the investigator in recording all reactions, the more complex are the patterns likely to be. When a definite number of plaques, such as 50, is taken as the basis for identification of a strain, it is not surprising that identical results are not always obtained by different workers studying the same strains, or even by the same worker studying the same strain at different times. Williams and Rippon (19) studied the variability of strains considered to be epidemiologically the same and suggested that in those strains tested on the same day, two strong differences, namely, the complete absence in one strain of lysis by two phages giving strong lysis in the other, may be regarded as indicating a distinct population. We have accepted this suggestion in principle and have followed it, in general, in reporting our results. The multiplicity and complexity of these patterns have made it difficult to report results of phage-typing investigations. Through the frequency of association of lytic reactions, Williams and co-workers (20) divided the staphylococci into three broad groups. The International Staphylococcal Bacteriophage Typing and Reference Centre at Colindale has suggested a slightly modified system of grouping which has

been followed in this report. This grouping as it relates to the phages used in this study are as follows:

Group I—Phages 29, 29A, 52, 52A, 79.
Group II—Phages 3A, 3B, 3C, 51, 55.
Group III—Phages 6, 7, 42B, 42E, 47, 47B, 47C, 53, 54, 70, 73, 75, 76, 77.
Group IV—Phage 42D.
Miscellaneous—Phages 31, 42C*, 44, 44A, 47A, 57, 81.

The reading of the plates is sometimes difficult, particularly when the plaques are small and the culture has grown luxuriantly. The use of a stereoscopic microscope with the light transmitted from a mirror placed, on the bench, equidistant from the microscope and the illuminator, namely, Landy's (11) method of distinguishing V from W forms of *S. typhi*, greatly facilitated the reading of the plaques. It is recommended that this method be used routinely to eliminate differences in results obtained by different investigators.

Antibiotic-sensitivity Tests

All cultures were tested for their sensitivity to penicillin, chlortetracycline, oxytetracycline, chloramphenicol, streptomycin, and erythromycin by the serial tube-dilution method. To nine Wassermann tubes each containing 0.5 ml. of a series of double dilutions of the antibiotic in Bacto-Penassay broth and to one control tube containing broth without antibiotic was added one drop of a 1:500 dilution of an 18-hr. Penassay broth culture of the test organism. The tubes were incubated at 37° C. for 18 hr. and examined for growth. The tube with the lowest concentration of the drug which showed no visible growth was read as the minimum inhibitory concentration of the drug for the test organism. Each culture was also tested by the disk method for sensitivity to the six antibiotics and to neomycin as well. Plates of Bacto-tryptose agar were seeded with 0.04 ml. of an 18-hr. Penassay broth culture of the test organism, diluted to a density equal to that of a No. 2 McFarland tube, and the inoculum spread evenly over the entire surface of the agar to give confluent growth. Bacto-sensitivity disks of the seven antibiotics were immediately placed on the surface, spaced as evenly as possible. The plates were then incubated at 37° C. for 18 hr. and examined for zones of inhibition. A clear zone of any size around a disk was considered as 'inhibition'. Only one concentration of each antibiotic was used in this test—penicillin (1 unit), chlortetracycline (10 μgm.), oxytetracycline (10 μgm.), chloramphenicol (10 μgm.), streptomycin (10 μgm.), erythromycin (1 μgm.), and neomycin (10 μgm.). The disk test was only run as a rough check on the tube-dilution method and results reported here are based on tube tests. The only exception to this is in the case of neomycin which was tested by the disk method alone.

There is no absolute or universally accepted level of drug concentration which can be used to classify an organism as resistant or sensitive to a particular drug. Different interpretations are placed on the results of these laboratory

**While the special committee of the International Committee has suggested that phage 42C be placed in the "Miscellaneous" group, we have in this study found it so commonly associated with the Group III phages that we have included it in the Group III patterns.*

sensitivity tests by different investigators. Many, moreover, fail to state in their reports the values used for their interpretations. In this report, the authors have, with a few minor changes, used the values recommended by the D.V.A. workers (1, 13) who have attempted to select these values on the basis of their relationship to clinical treatment. The values which we adopted were as follows:

Antibiotic	Sensitive (S)	Moderately resistant (MR)	Resistant (R)
	Organism inhibited (tube-dilution method) by		
Penicillin	0.5 Units/ml.	5 U./ml.	> 5 U./ml.
Chlortetracycline	1.0 μgm./ml.	25 μgm./ml.	>25 μgm./ml.
Oxytetracycline	1.0 μgm./ml.	25 μgm./ml.	>25 μgm./ml.
Erythromycin	1.0 μgm./ml.	25 μgm./ml.	>25 μgm./ml.
Chloramphenicol	10.0 μgm./ml.	50 μgm./ml.	>50 μgm./ml.
Streptomycin	5.0 μgm./ml.	50 μgm./ml.	>50 μgm./ml.
Neomycin (disk)	10.0 μgm./ml.		>10 μgm./ml.

Results

Of the dressings taken from the first 1400 patients after what Howe (8) considered 'clean' operations, 220 (15.7%) showed the presence of *S. aureus*. One of the authors (R.H.E.) reviewed the charts of all patients with positive dressings and attempted to assess the clinical significance of the presence of the staphylococci. One hundred and fourteen of the wounds contaminated with *S. aureus*—8% of the 1400 dressings examined—were considered to be 'septic' or 'clinically infected'. One hundred and six (7.5%) were apparently what Williams and Miles (18) describe as 'silently infected', that is, contaminated but showing no definite evidence of clinical disease. These authors claimed that this contamination resulted in some delay in the healing of wounds. This is most difficult to assess and we have made no attempt to do so.

Phage-typing

In Table I are shown the results of our phage-typing tests on 516 cultures. Only 24 (4.6%) were untypable with the phages used; 464 (90%) were typable at R.T.D. and 28 (5.4%) were typable only in higher concentrations than R.T.D. Group III was the predominant type, 47.1% of all strains examined falling into this category. With one exception, this 'type' was more common in the hospital patients and personnel than in the group of student nurses before their exposure to the hospital environment, who might be taken as representative of the population outside the hospital. The one exception was the group of patients with typical staphylococcal infections, other than infected wounds. Most of these infections were carbuncles, boils, and breast abscesses. Type 81 predominated in this group of patients, causing 50% of the infections. This interesting type is shown in a separate column in the table. Dr. Williams placed this phage in the Miscellaneous Group, because

TABLE I

PHAGE-GROUPING OF *S. aureus* CULTURES ISOLATED FROM DIFFERENT SOURCES

Source		Phage group I	II	III	IV	81	Misc.*	Untypable	Total
I. Patients with boils, abscesses, etc.		2	4	13	0	35	13	3	70
	%	2.8	5.7	18.5	—	50.0	18.5	4.3	
II. Patients with clinically infected wounds		1	4	78	0	9	15	7	114
	%	0.9	3.5	68.4	—	7.9	13.1	6.1	
III. Patients—wounds not clinically infected		0	7	72	0	8	15	4	106
	%	—	6.6	67.9	—	7.5	14.1	3.8	
IV. Patients—wounds (histories not available)		0	4	35	0	14	15	3	71
	%	—	5.6	49.3	—	19.7	21.1	4.2	
V. Student nurses (51)† on starting their training		7	14	14	1	0	15	4	55
	%	12.7	25.4	25.4	1.8	—	27.2	7.3	
VI. Same group of student nurses (54)‡ after three months of training		6	15	21	2	3	11	3	61
	%	9.8	24.6	34.4	3.3	4.9	18.0	4.9	
VII. Nurses—on routine active ward duty		0	2	9	0	8	4	0	23
	%	—	8.7	39.1	—	34.8	17.4	—	
VIII. Ward (pediatric) air		4	1	1	0	5	5	0	16
	%	25.0	6.2	6.2	—	31.2	31.2	—	
Totals		20	51	243	3	82	93	24	516
	%	3.9	9.9	47.1	0.6	15.9	18.0	4.6	

**Misc.* = *Includes not only those strains lysed by phages of the 'Miscellaneous' group but also those giving reactions by phages in different groups, viz. I/III; I/III/M; I/II/IV/M I/III/IV/M; III/M; II/III/M, and II/III/IV/M.*

†*Four students carried two types of S. aureus.*

‡*Seven students carried two types of S. aureus.*

of its action on Group I and Group III strains. On the other hand, there is a very close similarity between this phage and Rountree's phage '80' which has been placed in Group I. Dr. Rountree (15) in a study of 226 strains reacting with either phage 80 or 81 or both, found that 153 (67.7%) gave the same reactions with the two phages at R.T.D. while 29 reacted with 80 only, and 44 with 81 only. It is evident from the investigations of both Williams and Rountree (personal communications) that the two phages are different but it is also clear from Rountree's observations that the majority of strains, described by her as type '80', using phage 80 alone would undoubtedly have been classified as type 81, if phage 81 had been used instead of phage 80. We have not had the opportunity of investigating phage 80, but two cultures received from Dr. Rountree as type '80' gave identical reactions to our '81' strains. There is a great likelihood then that many of the '81' strains described in this report are similar, if not identical with the 52AV (new type '80') strains described by Isbister, Durie, Rountree, and Freeman (9) as the causative organism in serious epidemics of staphylococcal infection of the newborn in a number of hospitals in Australia.

While Group III and type 81 strains were predominant (72%) among the hospital strains, and Group I (2%) and Group II (6%) strains were relatively uncommon, type 81 was not found among the student nurses starting their training. Group III was less common (25%), while Group II (25%) and Group I (12%) were relatively more common.

Of 95 student nurses examined on starting their training, before exposure to the hospital environment, 51 (53.7%) were found to be carrying coagulase-positive staphylococci in their throats and/or noses: four of these students were carriers of two different types of *S. aureus*. These same student nurses were examined three months later. While much of this training period was spent in the classroom, there was some training given on the wards, with exposure to the hospital flora. At this second examination, 54 (56.8%) were carrying *S. aureus*; two types were isolated from seven. Sixteen who were positive on first examination were negative three months later, while 19 negative on first examination were carriers at the second examination; 35 were positive at both examinations. Of these 35, nine showed a change of type; at least the cultures isolated at the two periods were different. It is quite possible that the type found at the second examination was present originally but was not isolated for study; similarly, the type originally found may have been present at the time of the later examination but was not isolated for study. While this short period of time between examinations and very limited exposure to the hospital flora revealed no striking differences in the staphylococcal flora of these student nurses (Tables I, II, III, and IV), there is a marked difference between their flora and that of the group of 23 regular nurses who had been on the wards for a long time. In this latter group 35% were carrying type 81.

A rather interesting observation was the similarity of the type-distribution of the staphylococci in the group of patients with clinically infected wounds and in the group with the 'contaminated' but not infected wounds. No particular group appears to be specifically septic for wounds.

TABLE II

PREDOMINANT PHAGE PATTERNS IN GROUPS I AND II OF *S. aureus* FROM DIFFERENT SOURCES

Source	Group I		Group II	
	No.	Patterns	No.	Patterns
I. Boils, abscesses, etc.	2	52/52A (2)	4	3A/3B/3C/51 (4)
II. Clinically infected wounds	1	52A	4	3A; 3A/3B/3C/51; 3A/3C/51; 3B/3C
III. Wounds—not clinically infected	0		7	3A/3B/3C/51 (3); 3A (2)
IV. Wounds—(histories not available)	0		4	3B/3C (2)
V. Student nurses—on starting training	7	29 (4); 52/52A (3)	14	3A/3B/3C/51 (6); 3C/55 (3); 3B/3C/55 (3)
VI. Same group of nurses—three months later	6	29 (4); 29/29A (2)	14	3A/3B/3C/51 (4); 55 (3); 3A/51 (3)
VII. Nurses—on routine active ward duty	0		2	3A/3C (2)
VIII. Ward air	4	52/52A (4)	1	3A

TABLE III

PREDOMINANT PHAGE PATTERNS IN GROUP III OF *S. aureus* FROM DIFFERENT SOURCES

Source	No.	Patterns
I. Boils, abscesses, etc.	13	7/42C/47/47B/47C/53/54/73/76/77 (54%)
II. Clinically infected wounds	78	53/54/70/76/77 (45%); 7/42C/47/47B/47C/53/54/73/76/77 (35%)
III. Wounds—not clinically infected	72	7/42C/47/47B/47C/53/54/73/76/77 (58%); 53/76/77 (22%)
IV. Wounds—(histories not available)	35	53/54/75/76/77 (46%); 7/42C/47/47B/47C/53/54/73/76/77 (26%); 47/53/54/75/76/77 (23%)
V. Student nurses—on starting training	14	76/77 (36%); 7/42C/47/47B/47C/53/54/73/76/77 (14%)
VI. Same group of nurses—three months later	21	53/76/77 (52%); 7/42C/47/47B/47C/53/54/73/76/77 (14%)
VII. Nurses—on routine active ward duty	9	47/53/54/75/76/77 (44%); 7/42C/47/47B/47C/53/54/73/76/77 (22%)
VIII. Ward air	1	47/47B/47C/75/76

TABLE IV

PREDOMINANT PHAGE PATTERNS IN THE MISCELLANEOUS AND MIXED GROUPS

Source	No.	Patterns
I. Boils, abscesses, etc.	48	81 (73%); 31/44/57/81 (6%); 7/31/44/44A/81 (6%)
II. Clinically infected wounds	24	81 (38%); 47C/52/52A/81 (29%)
III. Wounds—not clinically infected	23	81 (35%); 52/52A/81 (22%)
IV. Wounds—(histories not available)	29	81 (48%); 7/29/29A/31/42B/42C/42D/42E/44A/47/47B/47C/52A/81 (14%); 47C/52/52A/81 (10%)
V. Student nurses—on starting training	16	29A/31/44/44A (19%); 52/81 (12%)
VI. Same group of nurses—three months later	14	81 (21%); 42B/47C/81 (14%); 31/44/52A (14%)
VII. Nurses—on routine active ward duty	12	81 (67%); 47C/52/52A (17%)
VIII. Ward air	10	81 (50%); 52/52A/81 (40%)

The predominant patterns found in the different groups from the eight sources studied are shown in Tables II, III, and IV. One or two patterns seem to predominate in each group, regardless of source.

Antibiotic-sensitivity Tests

The antibiotic-sensitivity of the cultures is shown in Tables V and VI. Of the 552 cultures studied, only 39% were sensitive to penicillin, 54–57% to the tetracyclines, and 60% to streptomycin, while 100% were sensitive to neomycin, 99% to erythromycin, and 95% to chloramphenicol. There was, however, a striking difference in the sensitivity of strains isolated from the student nurses before exposure to the hospital environment and that of the hospital strains (the three first horizontal columns of Table V). More than

TABLE V

ANTIBIOTIC-SENSITIVITY TESTS

Source	No. cultures		Pen.			Chlortet.			Oxytet.			Erythro.			Chloram.			Strepto.			Neo.	
			S	MR	R	S	MR	R	S	MR	R	S	MR	R	S	MR	R	S	MR	R	S	R
I Patients in hospital	369		86	25	258	157	140	72	152	22	195	368	1	—	360	6	3	176	14	179	369	—
		%	23.3	6.8	69.9	42.5	37.9	19.5	41.2	5.9	52.8	99.7	0.3	—	97.5	1.6	0.8	47.7	3.8	48.5	100	—
II. Nurses—on routine active ward duty	23		7	—	16	18	3	2	18	—	5	23	—	—	19	4	—	15	3	5	23	—
		%	30.4	—	69.5	78.2	13.0	8.7	78.2	—	21.7	100	—	—	82.6	17.4	—	65.2	13.0	21.7	100	—
III. Ward (pediatric) air	16		2	—	14	4	6	6	4	—	12	16	—	—	10	1	5	16	—	—	16	—
		%	12.5	—	87.5	25.0	37.5	37.5	25.0	—	75.0	100	—	—	62.5	6.2	31.2	100	—	—	100	—
IV. Student nurses—on starting training	72		66	2	4	72	—	—	66	6	—	72	—	—	69	2	1	67	4	1	72	—
		%	91.6	2.8	5.5	100	—	—	91.6	8.3	—	100	—	—	95.8	2.8	1.4	93.0	5.5	1.4	100	—
V. Same group of nurses—three months later	72		56	—	16	61	5	6	61	—	11	71	—	1	71	1	—	60	2	10	72	—
		%	77.8	—	22.2	84.7	6.9	8.3	84.7	—	15.3	98.6	—	1.4	98.6	1.4	—	83.3	2.8	13.9	100	—
Totals	552		217	27	308	312	154	86	301	28	223	550	1	1	529	14	9	334	23	195	552	—
		%	39.3	4.9	55.8	56.5	27.9	15.6	54.5	5.1	40.4	99.6	0.2	0.2	95.8	2.5	1.6	60.5	4.2	35.3	100	—

TABLE VI

ANTIBIOTIC-SENSITIVITY TESTS

Phage group	No. cultures		Pen. S	Pen. MR	Pen. R	Chlortet. S	Chlortet. MR	Chlortet. R	Oxytet. S	Oxytet. MR	Oxytet. R	Erythro. S	Erythro. MR	Erythro. R	Chloram. S	Chloram. MR	Chloram. R	Strepto. S	Strepto. MR	Strepto. R	Neo. S	Neo. R
Group I	25		21	—	4	21	3	1	21	—	4	25	—	—	23	1	1	24	1	—	25	—
		%	84.0	—	16.0	84.0	12.0	4.0	84.0	—	16.0	100	—	—	92.0	4.0	4.0	96.0	4.0	—	100	—
Group II	60		59	1	—	60	—	—	56	4	—	60	—	—	57	2	1	57	3	—	60	—
		%	98.3	1.7	—	100	—	—	93.3	6.6	—	100	—	—	95.0	3.3	1.7	95.0	5.0	—	100	—
Group III	262		52	18	192	97	96	69	94	17	151	262	—	—	256	3	3	71	4	187	262	—
		%	19.8	6.9	73.3	37.0	36.6	26.3	35.9	6.5	57.6	100	—	—	97.7	1.1	1.1	27.1	1.5	71.4	100	—
Group IV	3		3	—	—	3	—	—	3	—	—	3	—	—	2	1	—	3	—	—	3	—
		%100		—	—	100	—	—	100	—	—	100	—	—	66.7	33.3	—	100	—	—	100	—
Type '81'	84		—	4	80	30	42	12	31	1	52	84	—	—	77	4	3	74	7	3	84	—
		%	—	4.7	95.2	35.7	50.0	14.3	36.9	1.2	61.9	100	—	—	91.7	4.7	3.6	88.1	8.3	3.6	100	—
Miscellaneous	94		61	4	29	79	11	4	74	6	14	92	1	1	91	2	1	86	5	3	94	—
		%	64.9	4.2	30.8	84.0	11.7	4.2	78.7	6.4	14.9	97.9	1.0	1.0	96.8	2.1	1.0	91.5	5.3	3.2	100	—
Untypable	24		21	—	3	22	2	—	22	—	2	24	—	—	23	1	—	19	3	2	24	—
		%	87.5	—	12.5	91.6	8.3	—	91.6	—	8.3	100	—	—	95.8	4.2	—	79.1	12.5	8.3	100	—

90% of all the strains isolated from these students (Table V, column 4) were sensitive to all the antibiotics, while only 23% of the hospital strains were sensitive to penicillin, 44% to chlortetracycline, 42% to oxytetracycline, and 50% to streptomycin.

In Table VI are shown the sensitivities of the different phage-groups to the antibiotics. Most of the Group I and II strains, the three strains of Group IV, and the untypable strains were sensitive to all the antibiotics. The strains in the Miscellaneous Group (exclusive of type 81) were slightly more resistant, while the Group III strains, the predominant group in the hospital, were very much more resistant to penicillin, the tetracyclines, and streptomycin. The type 81 strains were also resistant to penicillin and almost 65% of them were either resistant or moderately resistant to the tetracyclines, but approximately 90% were sensitive to streptomycin as compared with only 27% of the Group III strains.

Discussion

This report confirms what many (3, 5, 14, 16, 20) have found, namely, that of the cultures of *S. aureus* isolated in the hospital, Group III strains are predominant. Moreover, as found by these authors the strains of this group are, in general, more resistant to the commonly used antibiotics, penicillin, chlortetracycline, and oxytetracycline. The conclusion that it is the increased resistance of these strains that has led to their predominance in the hospital where these antibiotics are in constant use would seem to have some justification. It has been generally assumed that the extensive use of antibiotics in the hospital has inhibited the sensitive strains brought into the hospital from outside, and allowed the resistant strains to develop, rather than that the sensitive strains have developed resistance of their own. In favor of this view is the finding of Group III strains predominant in the healthy hospital personnel and patients not under antibiotic treatment. The gradual changeover, in the incoming patient or staff member, from a staphylococcal flora in which the sensitive strains of Group I and Group II are prominent, to that in which the resistant strains of Group III predominate also indicates the acquisition of new types rather than the developing resistance of the original types. Gould (7), however, has recently reported the development *in vitro* of penicillinase-producing Group III variants in penicillin-sensitive strains of Group I and Group II parent cultures. It is possible therefore that several mechanisms are responsible for the predominance of the resistant Group III strains in the hospital staphylococcal flora.

An interesting observation, in this investigation, was the finding of a new type '81', particularly commonly associated with boils and abscesses. Since the discovery of this type, we have found it to be very common in a number of other hospitals in Canada. The close similarity between Rountree's type 80 strains and our '81' strains point to this being a common hospital 'epidemic'

strain. Moreover, it appears unreasonable that these two closely related types should be placed in different groups, the '80' strains in Group I, and the '81' in the Miscellaneous Group.

The successful typing of 95% of the cultures studied calls for some comment, since in most investigations reported, only from 60 to 80% of the cultures could be typed. The use of phages at dilutions lower than R.T.D. enabled us to type 24 (4.6%) strains, and the discovery of a new phage '81' allowed us to type an additional 16% of strains, while the use of 32 phages permitted the typing of more strains than in those series, where in general, fewer phages were used.

The results reported stress the importance and necessity of antibiotic-sensitivity testing of staphylococci isolated in hospital before antibiotic therapy is initiated. This is less important, however, in staphylococcal infections developing outside the hospital, as the majority of the infecting strains are still likely to be penicillin-sensitive, and penicillin remains the drug of choice for the treatment of infections caused by sensitive strains.

Despite the demonstration by Lepper *et al.* (12) of the rapidly developing resistance to erythromycin of staphylococci in a hospital where erythromycin was substituted for penicillin in the routine treatment of the patients, after several years of restricted use of this drug in the Ottawa hospital studied, there was no detectable increase in resistance of the staphylococci to this drug. The increased antibiotic-resistance of the staphylococci causing infections in the hospital poses a serious problem to the physician, and the continued restricted and intelligent use of this useful drug should be more strictly adhered to. The fear of blood dyscrasias has undoubtedly seriously curtailed the use of chloramphenicol. It is interesting to note that the staphylococci in this series had developed little resistance to this drug.

Howe (8) considers that on a well-regulated surgical service the rate of postoperative wound infections should not exceed 1 or 2% of the clean operations. The finding of an 8% postoperative wound infection rate in our study would indicate the need for more attention to good aseptic surgical and nursing techniques in this hospital, and this probably applies generally. Perhaps less dependence should be placed on the antibiotics to cover up carelessness in the operation room.

Acknowledgments

The authors wish to thank Miss D. E. Helmer and Miss D. Allard, of the Laboratory of Hygiene, for their technical assistance and Dr. R. E. O. Williams, Colindale, for the many phages and their propagating strains that he gave to us and for his study of our new type '81' phage.

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AN INVESTIGATION OF "A" SUBSTANCE IN T.A.B. VACCINE DILUTED WITH SALINE OR TOXOID[1]

By H. C. Plummer and M. H. Brown

Abstract

"A" substance, comparable to that found in human red blood cells, has been reported to be present in toxoid and vaccine made on media containing tryptic or peptic digests of animal tissue. Our tests were carried out with T.A.B. vaccine produced on (1) beef leg infusion containing 1% Bacto peptone and 3% Bacto agar, and (2) on beef heart infusion containing 1% N-Z case (tryptic digest of casein) and 3% agar. When either type of vaccine was diluted with saline, for human use, no trace of "A" substance was found. When T.A.B. vaccine was diluted with tetanus toxoid (T.A.B.T.) or with tetanus and diphtheria toxoids (T.A.B.T.D.), considerable "A" substance was present if the toxoids were produced on hog stomach autolyzate medium (peptic digest of hog stomach tissue). However, when the vaccines were diluted with toxoids produced on N-Z case medium, no "A" substance was found.

Introduction

Reports (3, 5) have been published indicating the presence of "A" substance, comparable to that in human red blood cells, in toxoids, and vaccines produced on media containing peptic or tryptic digests of animal tissue. It is thought that group-A antibodies produced in group-O subjects by the injection of such antigens may be the source of some transfusion reactions (1, 2, 4). Digests of casein have recently largely replaced peptones of animal origin in the preparation of diphtheria and tetanus toxoids (4). With these things in mind we sought for a suitable casein digest medium upon which strains of salmonella would grow abundantly. Thirty-two different kinds of medium were tested and the medium of choice consisted of beef heart infusion, 0.5% NaCl, 1% N-Z case (tryptic digest of casein from the Sheffield Chemical Company Inc.), and 3% Bacto agar. On this medium the three strains of salmonella used in the tests grew as well as on the usual medium composed of beef leg infusion, 0.5% sodium chloride, 1% Bacto peptone, and 3% Bacto agar. Antigenic tests carried out in mice showed no significant difference in the protection afforded by vaccine from either type of medium. Agglutinin–absorption tests were used to detect the presence of "A" substance in media, various components of media, and in the finished antigens.

Methods

Preparation of Vaccine

Vaccines were made respectively from three strains of salmonella, namely *S. typhosa* "Rawlings rejuvenated," *S. paratyphi* (*S. paratyphi* A), "Mears", and *S. schottmuelleri* (*S. paratyphi* B) "US 41 H6". Separate vaccines were made from each strain. The bacteria were grown on medium composed of beef heart infusion, 1% N-Z case, 0.5% sodium chloride, and 3% Bacto agar.

[1]*Manuscript received October 11, 1955.*
Contribution from the Connaught Medical Research Laboratories, University of Toronto, Toronto, Ontario. Aided by a grant from the Defence Research Board of Canada.

The 18 hr. growth was harvested by being washed from agar with physiological saline, 20 ml. being added to each Roux bottle of *S. typhosa* and 40 ml. per bottle of the other strains. The suspensions were killed by being heated in a water bath at 56° C. for one hour, then phenol was added as a preservative to give a concentration of 0.4%. Another set of vaccines was similarly produced on medium composed of beef leg infusion, 1% Bacto peptone, 0.5% sodium chloride, and 3% Bacto agar. After harvesting, the vaccine from each strain contained approximately 30 billion bacteria per milliliter. When a lot of T.A.B. was diluted with saline for human use it contained 1500 million *S. typhosa*, 500 million *S. paratyphi* A, and 500 million *S. paratyphi* B per milliliter. When diluted with toxoid to make T.A.B.T. or T.A.B.T.D. vaccine, there were 750 million *S. typhosa*, 250 million *S. paratyphi* A, and 250 million *S. paratyphi* B per milliliter.

Several lots of cholera vaccine produced by growing *V. cholerae*, strains 35 and 41 respectively, on 1% of a commercial peptone, which has been designated Peptone X, with 3% Bacto agar were included in the tests. These strains were also grown on medium composed of 1% N-Z case with 3% Bacto agar. When prepared for human use, the killed vibrios in approximately equal numbers were diluted with saline to a final concentration of 8000 million per milliliter (a dilution of approximately 1 : 6).

Absorption of Anti-A Serum

The anti-A serum (Ortho Pharmaceutical Corporation Ltd.) was diluted 1 : 10 with physiological saline and 0.04 ml. placed in each agglutination tube. An equal volume of material to be tested for the presence of "A" substance was added to the tube containing the serum, mixed, and left at room temperature for two hours. If concentrated vaccine (not diluted for human use) was used, 0.06 ml. of serum and of vaccine respectively were mixed, left at room temperature for two hours, centrifuged, and the supernatant fluid poured into a clear tube to be used as the absorbed serum.

Preparation of Red Cell Suspension

Five milliliters of blood from a group-A donor were placed in a tapered 15 ml. centrifuge tube containing 0.5 ml. of sterile 10% sodium citrate solution. The citrated blood was centrifuged at moderate speed for 15 min. and the plasma pipetted off. The red cells were washed by adding saline to equal the original volume of blood, centrifuging for 10 min., and decanting the supernatant fluid. Two further similar washings of cells took place. A 2% suspension was made by adding 0.2 ml. of packed washed red cells to 9.8 ml. of physiological saline. When the cell suspensions were used on the day of drawing the blood and on the following day, no difference was noted in their agglutinability. These suspensions of red blood cells were never used after the second day.

Test for "A" Substance

To each tube containing 0.08 ml. of absorbed serum was added 0.04 ml. of 2% suspension of group-A red cells. These materials were mixed and left at

room temperature for two hours. Readings of agglutination of red cells were made directly from the tubes and also after the contents had been emptied into well-slides. No absorption of anti-A serum was indicated by the formation of large aggregations of red cells, and partial absorption by small clumps of cells. Complete absorption was indicated by no aggregation or clumps of red cells. If the test material absorbed the antibodies from the serum it was assumed to contain "A" substance.

Results

When slight or no absorption of anti-A serum took place, the agglutination of the group-A red cells was easily detected in the agglutination tubes. However, when partial absorption occurred, agglutination of cells was more readily seen after the contents of the tube were poured into a well-slide. All readings in the table are from well-slides. Two group-A donors were used and it appeared that the cells of one were more readily agglutinable than those of the other. The results in the tables are taken from the tests using the more agglutinable red cells.

Of the various media tested, only two commercial peptones (designated as X and Y), one known to be composed chiefly of proteoses, indicated the presence of considerable amounts of "A" substance (Table I). A 5% solution of each of these peptones when diluted 1 : 1000 with saline still showed a trace of

TABLE I

PRESENCE OF "A" SUBSTANCE IN MEDIA, AQUEOUS SOLUTIONS OF INGREDIENTS OF MEDIA, AND IN CONCENTRATED VACCINES SUSPENDED IN PHYSIOLOGICAL SALINE

Material for absorption of serum*	Agglutination of group A† cells by absorbed sera	Amount of "A" substance
Beef leg infusion + 1% Bacto peptone	4+, 4+, 4+, 4+, 4+	0
Beef heart infusion + 1% N-Z case	2+, 4+, 4+	0
Beef heart infusion	4+, 4+	0
1% N-Z case	4+, 4+, 4+, 4+, 4+	0
1% N-Z amine A	4+	0
1% N-Z amine B	4+	0
1% N-Z amine E	4+	0
1% Bacto peptone	3+, 4+, 4+, 4+	0
1% commercial peptone X	0, 0, 0	Abundant
1% commercial peptone Y	0	Abundant
1% soybean peptone	4+, 4+, 4+	0
1% Bacto yeast extract	4+, 4+	0
Concentrated typhoid vaccine (B.P.)‡ 2 lots	1+, 0	Abundant
" " " (N-ZC)‡ 1 lot	0	Abundant
" paratyphoid A vaccine (B.P.) 2 lots	4+, 4+	0, 0
" " A " (N-ZC) 1 lot	3+	Slight
" paratyphoid B " (B.P.) 2 lots	3+, 3+	Slight, slight
" " B " (N-ZC) 1 lot	2+	Moderate

**Material: N-Z case, N-Z amines. Soybean peptone from Sheffield Chemical Co. Bacto products from Difco.*

†Agglutination of group A cells:

4+ = large clumps—no absorption of anti-A serum.
3+ = smaller " —slight " " " "
2+ = small " —moderate " " " "
1+ = very small " —considerable " " " "
0 = no clump —complete " " " "

Each figure with a plus sign, and each 0 represent one test.

‡B.P. = Bacto peptone; N-ZC = N-Z case.

"A" substance. Beef leg infusion (1 lb. meat per liter of water) containing 1% Bacto peptone, beef heart infusion (1 lb. heart per liter of water) either with or without 1% N-Z case, and 1% aqueous solutions of N-Z case, N-Z amine A, N-Z amine B, N-Z amine E, soybean peptone, Bacto peptone, and Bacto yeast extract did not contain any "A" substance as shown by our tests.

In order to test for "A" substance in concentrated vaccine, two lots of vaccine were made on Bacto peptone agar, and one lot on N-Z case agar for *S. typhosa*, *S. paratyphi* A, and *S. paratyphi* B respectively. The concentrated whole vaccine, the supernatant fluid of the centrifuged vaccine, and the thrice-washed bacterial cells of each vaccine were tested for "A" substance. The whole vaccine and the supernatant fluid of the three lots of typhoid vaccine each showed "A" substance. Only a slight trace of "A" was detected in the whole vaccine of paratyphoid A produced on N-Z case agar, but not in any of the other fractions tested. With paratyphoid B vaccine, a slight trace of "A" substance was detected in the whole vaccine and supernatant fluids but not in the washed bacterial cells. However, it seems that only a small concentration of "A" substance was present, since none could be detected when the vaccines or supernatants were diluted 1 : 10 with saline. Tests on T.A.B. vaccine diluted for human use are shown in Table II. Thirteen lots diluted with saline to contain the required number of bacteria (1500 million T, 500 million A, 500 million B) did not show any "A" substance. Eight of these lots were prepared from vaccine grown on Bacto peptone agar and five lots from N-Z case agar. On the other hand, 10 lots of T.A.B. vaccine diluted with tetanus toxoid, the latter being made on hog stomach autolyzate medium, showed large amounts of "A" substance. Traces were still evident when the T.A.B.T. was diluted 1 : 1600 with saline. Two other lots of T.A.B.T. in which the tetanus toxoid was made in N-Z case medium showed no "A" substance. Tests on T.A.B.T.D. indicated that when T.A.B. vaccine is

TABLE II

PRESENCE OF "A" SUBSTANCE IN ANTIGENS CONTAINING T.A.B. VACCINE OR CHOLERA VACCINE DILUTED FOR HUMAN USE

Vaccine	Peptone in medium for vaccine production	Fluid for dilution	No. lots	Amount of "A" substance present
T.A.B.	Bacto peptone	Saline	8	0
T.A.B.	N-Z case	Saline	5	0
T.A.B.T.	Bacto peptone	Tetanus toxoid (H.S.A.)*	10	Abundant
T.A.B.T.	N-Z case	Tetanus toxoid (N-ZC)†	2	0
T.A.B.T.D.	Bacto peptone	Tet. and diph. toxoids (H.S.A.)	10	Abundant
T.A.B.T.D.	Bacto peptone	Tet. and diph. toxoids (N-ZC)	14	0
T.A.B.T.D.	N-Z case	Tet. and diph. toxoids (N-ZC)	26	0
Cholera	Comm. pep. X‡	Saline	8	Abundant
Cholera	N-Z case	Saline	1	0

**Hog stomach autolyzate medium for toxoid production.*
†N-Z case medium for toxoid production.
‡Commercial peptone X.

suspended in toxoid produced in hog stomach autolyzate medium, "A" substance is present, but not when N-Z case is the medium used for the toxoid production. Thus when all components of a combined vaccine are produced on casein digest media, no "A" substance can be demonstrated in the product diluted for human use.

Eight lots of cholera vaccine, made on commercial peptone X containing 3% agar and diluted with saline to contain approximately 8000 million *V. cholerae* per milliliter, each contained "A" substance. A trace could still be shown when the vaccines were diluted 1 : 100 with saline. One lot made on N-Z case agar was free of "A" substance. This was the only such lot tested.

Discussion

Our tests indicated some absorption of antibodies from anti-A serum by concentrated typhoid or paratyphoid B vaccine. Two lots of each strain were made on Bacto peptone agar, and one lot on N-Z case agar. Only a trace of "A" substance was shown in one lot of concentrated paratyphoid A vaccine made on N-Z case agar, but none in two lots made on Bacto peptone agar. A similar amount of absorption was shown by the supernatant fluids from centrifuged samples of the whole vaccines containing "A" substance, but none by the thrice-washed bacterial cells. Since no absorption of serum occurred when the whole vaccines or supernatants were diluted 1 : 10 with saline there could not have been much "A" substance present. In fact, the absorption may have been a non-specific effect due to the large amount of protein present in the vaccine or supernatant. In support of this hypothesis, Crawford *et al.* (3) found that alcoholized T.A.B. vaccine, made from organisms grown on tryptic digest agar, inhibited both anti-A and anti-B serum *in vitro*. They concluded that the effect was non-specific. They also found that the supernatant fluid from the centrifuged vaccine showed the same non-specific inhibiting effect as the whole vaccine. Thrice-washed bacterial cells did not show this effect. They injected washed T.A.B. bacteria into one group-B, and into two group-O persons, but found no significant change in the agglutinative titer of the blood, one week after the second injection. We now propose, with outside assistance, to study the anti-A titer of a number of group-O subjects both before and after injection of T.A.B.T.D. made on N-Z case media. It is also proposed to take a similar group who have had their primary stimulus with T.A.B.T.D. and examine samples of sera before and after the booster dose of T.A.B.T.D. vaccine. This experiment should show if an amount of "A" substance, too small to be detected by *in vitro* tests, could stimulate the production of anti-A serum in the human body.

Since early lots of T.A.B.T. and T.A.B.T.D. were made from toxoids produced on hog stomach autolyzate medium it is probable that "A" substance was present in considerable amounts in the T.A.B.T.D. used in the work reported by Conroy *et al.* (2). It is also probable that "A" substance was present in the T.A.B.T. and tetanus toxoid given their subjects prior to the booster dose of T.A.B.T.D. This could account for the fact that following

the booster dose of 0.5 ml. of T.A.B.T.D., from 80 to 85% of 20 servicemen possessed immune anti-A serum. For the past two years, in our laboratories, all vaccines containing T.A.B. as a component, as well as cholera vaccine, have been made on N-Z case media. Such products seem to be at least relatively free of "A" substance.

Summary

1. Beef leg infusion containing 1% Bacto peptone, and beef heart infusion containing 1% N-Z case (tryptic digest of casein) seem to be devoid of "A" substance. These media formed the base for the agar used for production of T.A.B. vaccine.

2. One per cent aqueous solutions of N-Z case, N-Z amine A, N-Z amine B, N-Z amine E, soybean peptone, Bacto peptone, and Bacto yeast extract showed no "A" substance. Considerable amounts were found in two commercial peptones tested.

3. Two lots of concentrated typhoid vaccine made from Bacto peptone agar and one lot from N-Z case agar each showed "A" substance present. A small amount was also indicated in three similar lots of paratyphoid B vaccine. Only a trace was found in the paratyphoid A vaccine made on N-Z case agar, but not in two lots made on Bacto peptone agar. The supernatant fluid of each concentrated vaccine contained the same amount of "A" substance as the corresponding whole vaccine. No "A" substance was found in the thrice-washed bacterial cells of any of these vaccines. When the whole vaccines or supernatant fluids were diluted 1 : 10 with saline, no "A" substance could be detected.

4. T.A.B. vaccine diluted for human use with saline showed no "A" substance. When diluted with tetanus toxoid (T.A.B.T.) or with tetanus and diphtheria toxoids (T.A.B.T.D.), "A" substance was present if the toxoid was made on hog stomach autolyzate medium. No "A" substance was demonstrated when the toxoid was made on N-Z case medium.

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THE PRESERVATION OF BACTERIA[1]

By R. I. N. Greaves

Abstract

The method of centrifugal vacuum freezing together with the use of a drying medium consisting of three parts serum, one part broth, and 7½% glucose has been shown to be suitable for preserving bacteria in the dry state. The method, however, demands a very rapid speed of evaporation and this may lead to contamination of the apparatus and even cross-contamination of cultures. Also, since the temperature of drying is not very low the choice of drying medium is restricted because of the frothing of many media that would otherwise be suitable. To overcome these difficulties an apparatus was designed for drying at a temperature below −30° C. With this apparatus a highly effective drying medium containing no protein can be used. The drying medium recommended for use with this apparatus consists of equal parts of 10% dextran and 12% peptone, to which is added glucose 7½% (w/v) and a detergent "Triton W.R. 1339", 1/4000 (w/v).

The preservation of bacteria and viruses by freeze-drying is now a routine procedure in many laboratories. Several methods of drying have been published but few papers record the percentage survival of organisms immediately after drying and fewer still the long-term survival. For many purposes the production of a living culture after drying is adequate even when the number of organisms which have survived the procedure is only a minute fraction of the original culture, but there are occasions when the percentage survival is of very great importance, as when the production of a dried living vaccine is contemplated.

Fry and Greaves (4) make some definite recommendations as a result of observing the percentage survival of organisms both immediately after drying and at intervals of up to four years after drying. Seven-year and a few eight-year survivals obtained since their paper was published confirm that the good initial survival rates have been well maintained. These authors worked mainly with a coliform bacillus 'D201H' because it was moderately sensitive to drying and easy to count. Later they applied their results to the drying of more delicate organisms such as *Vibrio cholerae* and *Neisseria gonorrhoeae*. Most of the drying was done by the centrifugal method of Greaves (5) at a temperature of −7° C. but some experiments carried out at −80° C. and −180° C. showed that though lower temperatures gave slightly higher survival rates, the medium in which the organisms were suspended had a far greater effect on the survival. Amittedly the relatively high temperature of −7° C. precluded the use of certain media which would have frothed badly at this temperature.

The observation that the medium is more important than the drying temperature is in agreement with the results of Stamp (7), who found that all but the most sensitive organisms could be dried at room temperature, and

[1]*Manuscript received October 26, 1955.*
Contribution from the Department of Pathology, University of Cambridge, United Kingdom.

of Annear (1), who found high survival rates even with delicate organisms when a single drop of a strong suspension of the organism in 10% glucose broth was placed on a plug of freeze-dried 12% peptone and rapidly dried *in vacuo* without freezing.

The most important constituent of the drying medium appeared to be glucose at a concentration between 5 and 10%. The suggestion was made that glucose controlled the residual moisture content; in other words, organisms preserved best if they were neither too wet nor too dry. Other sugars, such as lactose, had a similar effect. Simple peptone broth containing 7½% glucose was found to be an admirable medium for drying 'D201H', but delicate organisms like *N. gonorrhoeae* and *V. cholerae* dried better when some protein was added. As an all-round drying medium a mixture of three parts serum to one part broth with 7½% glucose per 100 ml. was recommended.

Assuming that the glucose exerts its effect by controlling the residual moisture content it is probable that the broth works in two ways. Firstly, its surface wetting property allows more intimate contact between the organisms and the suspending medium. Secondly, peptone itself appears to be a good medium (1, 2). Delicate organisms seem to need some slight further protection, which they get from serum proteins.

The centrifugal method and the medium of Fry and Greaves (4) have been widely and successfully used, but there are occasions when they are not applicable. The centrifugal method relies for success on very rapid evaporation and experience has shown (3, 6) that there is a tendency for small particles to be carried away on the water vapor stream, leading to contamination of the apparatus and even cross-contamination of tubes. The evaporation rate can be slowed down and contamination avoided by plugging the tubes but this is liable to lead to frothing of the medium and the production of a scaly preparation.

Serum may also be troublesome in that some sera cause agglutination of the organism. Moreover, the use of serum for preparing vaccines for human inoculation is definitely contraindicated, because homologous serum may give rise to jaundice in the recipient, and heterologous serum may cause allergic manifestations.

Rather than slow the speed of evaporation by obstructing the flow of water vapor, it would be better to refrigerate the ampoules during drying. If this were done the risk of contamination would be eliminated and there should be no trouble from frothing, thus allowing a much wider choice of drying media. An apparatus was therefore constructed in which the ampoules could be refrigerated during drying. The object of the present investigation was to determine whether the broth used in the original drying medium could be replaced by a simple surface wetting agent and whether the peptone in the broth was important. An additional object was to discover whether the protein in the original medium could be replaced by a non-antigenic polysaccharide of the dextran type and thus avoid the risk of allergy and

homologous serum jaundice when the material was injected into man. The discovery of a drying medium which could be used for drying living vaccines for human injection was the ultimate objective.

The Drying Apparatus

(*a*) *Refrigeration*

The refrigeration unit is a ¼ hp. Prestcold single stage compressor charged with 'Freon 12'. The evaporator coil is placed in a large Thermos flask of the type available on the war surplus market, which are reported to have been used by the Germans during the war for the storage of liquid oxygen for fueling V2 rockets. The flask has a capacity of approximately three gallons and is filled with alcohol as the secondary refrigerant. The refrigerator runs continuously and maintains the alcohol in the flask at −40° C. to −45° C.

In the bottom of the flask is a diaphragm-type petrol pump operated by a rod passing through the alcohol to a small electric motor mounted above the flask. This pump circulates the cold alcohol through the condenser and heater coils in the desiccator chamber. Since the motor is mounted outside the alcohol tank there is a minimal transfer of heat to the alcohol from this source.

(*b*) *Vacuum*

The desiccator chamber is pumped by an Edwards Speedivac Pump Model 1S50. Vacuum measurement is by a Pirani type gauge.

The vacuum entrance to the desiccator is through the tufnol end plate and is just behind the condenser coil. A small vacuum tap 'H' serves to admit air slowly at the end of desiccation.

(*c*) *Desiccator Chamber*

The chamber is made from a length of steel tube, 8 in. internal diameter by 18 in. long. The wall of the tube is ¼ in. thick. The tube is mounted at an angle of 10° to the horizontal sloping to the front. This ensures that during prefreezing the material freezes as a wedge and at the end of drying, when the ice on the condenser is thawed, the water will drain out of the front of the chamber.

Two rings (X in Fig. 1) are welded round the chamber one inch from each end. These preserve the shape of the chamber while the two ends are machined, and bolts passed through the ring at the far end of the chamber are used to attach the tufnol end plate.

Tufnol was chosen for this end plate because of its heat insulating properties. It is 1 in. thick and through it pass all the refrigerator pipes and the vacuum connection. These are vacuum sealed to the plate with trapped 'O' rings. An 'O' ring in a groove cut on the front of the plate makes the vacuum seal with the cylinder. The door is a 1 in. thick steel plate and is hinged, the vacuum seal again being made on a recessed 'O' ring; this door is tightened down with finger bolts to the ring welded to the chamber.

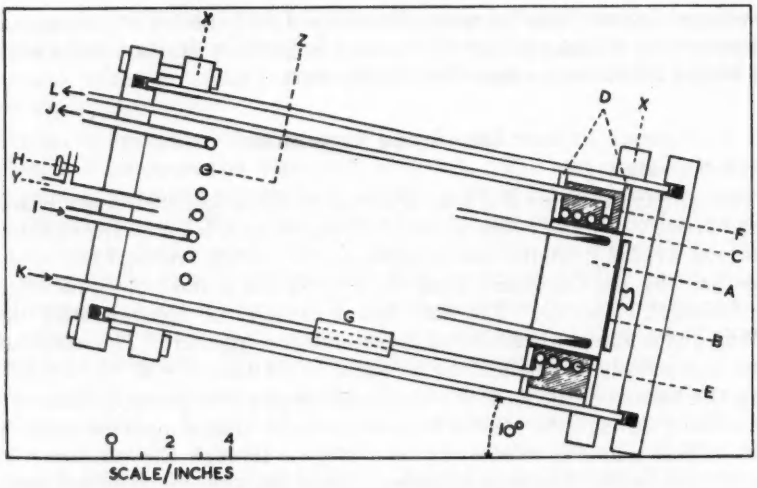

FIG. 1. Drawing of vacuum chamber.

A. Aluminum heated block drilled to take 78 tubes of culture.
B. Electric heater element.
C. Brass tubes surrounding aluminum block A.
D. Hexagonal tufnol plates supporting brass tube C.
E. Copper tubing for circulating refrigerant.
F. Glass wool lagging.
G. Electric heater element.
H. Vacuum let down tap.
I. Alcohol inlet pipe to condenser coil.
J. Alcohol outlet pipe from condenser coil.
K. Alcohol inlet pipe to heater head.
L. Alcohol outlet pipe from heater head.
X. Steel support rings.
Y. Vacuum connection.
Z. Condenser coil.

The heater head is more complex. It consists of an aluminum block 'A' 4½ in. in diameter, by 2 in. thick. It is drilled with 78 holes to fit the drying tubes which are 6 mm. in diameter. On the base of the block is mounted an electric iron heating element 'B' which is used for warming the block to room temperature at the end of drying.

The aluminum block is made a loose fit in the brass tube 'C'. This brass tube is located in the center of the desiccator by two hexagons of tufnol 'D' and round it is wound a coil of ⅜ in. copper pipe 'E' over which is placed a layer of glass wool 'F' as a heat insulator.

On the cold alcohol pipe leading to the heater head is placed an electric heating element 'G' which can be used to raise the heater head temperature if necessary. In fact this is seldom used because the heat losses of the head are such that it is always 5° C. hotter than the condenser surface and this differential has been found to give an adequately short drying time for the small quantity of culture required in each tube.

All temperatures are taken electrically with copper constantan thermocouples. Any six of these can be recorded simultaneously on a Cambridge Instrument Company's Recording Galvanometer.

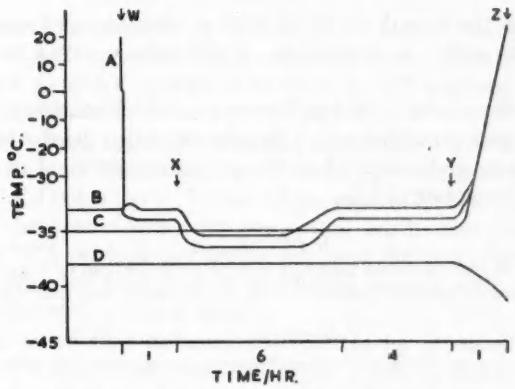

FIG. 2. Typical temperature curve during drying.

A. Temperature of culture suspension.
B. Temperature of alcohol leaving heater head.
C. Temperature of alcohol entering heater head.
D. Evaporator temperature.

(*d*) *Procedure*

The refrigerator is kept constantly running so that the alcohol flask is always at −45° C. The circulating pump is started and cold alcohol is circulated through both the condenser coil and the heater head. When after about two minutes the temperature of the condenser has fallen to −30° C. the chamber is evacuated so as to minimize loss of cold while the heater head is cooling. This takes about one hour, when the alcohol temperature will have risen and stabilized at −40° C., the evaporator coil will be at −38° C. and the heater block at −34° C.

Air is now admitted, the aluminum block removed, the tubes of culture, up to 78, placed in it, and the whole replaced in the chamber. Freezing occurs within 1 min. but is allowed to continue for at least 20 min., when the chamber is evacuated. When a pressure of 1 mm. Hg is reached the culture temperature falls until it is within a degree of the evaporator temperature. That drying is occurring fairly rapidly is shown by the fact that the alcohol is now leaving the heater head at a lower temperature than it is entering. The curves show that drying is complete in six hours but it is continued for a further four hours as a minimum, for convenience usually overnight. The alcohol circulation to the head is then stopped and the electric heater 'B' started. The heating current is regulated so that the block rises to room temperature in about one hour. Air is then admitted, the chamber is opened, and the tubes removed. The tubes are then constricted and sealed in high vacuum.

Fig. 2 shows typical recordings of temperatures during drying. For clarity the temperature scale between −30 and −40° C. has been expanded.

Tests of Drying Media

For the reasons already mentioned the coliform organism 'D201H' was used for these experiments. The organism was grown for 18 hr. on nutrient

agar slopes and the growth removed with a wire loop and emulsified in the drying medium under investigation. Each tube received 0.1 ml. of this suspension.

The first medium to be tried was 'Dextraven' which consists of 10% dextran in 5% glucose with no added salt. The product when dried was snowy white and of good form and texture but the percentage survival of the organism was very low, being only 1.7%.

TABLE I

SURVIVAL OF THE COLIFORM BACILLUS D201H AFTER DRYING IN VARIOUS MEDIA

	Medium	Percentage survival
A	Dextraven	1.7
B	Dextraven, Triton 1/4000 (w/v)	13.0
C	Horse serum three parts + broth one part, glucose 7½%	78.0
D	Horse serum, glucose 7½%, Triton 1/4000	71.0
E	Bovine albumin 25%, glucose 7½%, Triton 1/4000	70.0
F	Dextraven two parts + 12% peptone one part, glucose 7½%, Triton 1/4000	9.7
G	Dextraven one part + 12% peptone one part, glucose 7½%, Triton 1/4000	93.0
H	Dextraven one part + 12% peptone two parts, glucose 7½%, Triton 1/4000	64.0
K	Peptone 12%, glucose 7½%, Triton 1/4000	80.0

If part of the beneficial effect of broth in the medium is due to its surface wetting properties, it is reasonable to add some detergent to the Dextraven. 'Triton W.R. 1339'* at a dilution of 1/4000 (w/v) was recommended to us for this purpose by Dr. P. W. Muggleton of the Glaxo Laboratories, because it was reported to be less toxic to bacteria than 'Tween 80' and albumin would not be required as a detoxifying agent. The addition of Triton caused a substantial improvement in the percentage survival, which rose from 1.7% to 13.0%. This survival rate, however, was poor compared with the 78% survival in the standard serum–broth–glucose drying medium.

Tests were made to discover whether Triton could replace the broth in the original horse serum–broth–glucose mixture. Without broth the percentage survival fell from 78 to 66% and rose to 71% when Triton was added. Triton, therefore, does not seem to be a complete replacement for broth. However, replacement of the horse serum with 25% bovine albumin made very little difference, the percentage survival being 70% with 25% bovine albumin, 7½% glucose and 1/4000 Triton, and 71% with horse serum, 7½% glucose, and 1/4000 Triton. Obviously albumin is more suitable than serum in the medium because it does not agglutinate organisms; but it would be unsuitable

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for drying vaccines intended for the inoculation of man. The results summarized in Table I, A-E, considered in conjunction with Collier's (2) success in drying vaccinia virus for human inoculation in 5% peptone, suggest that peptone is highly desirable in the drying medium.

Table I, F-K, shows the result of drying experiments in which the proportions of peptone and Dextraven, were varied; all the mixtures contained 7½% glucose and 1/4000 (w/v) Triton. A survival of 80% with 12% peptone is as good as the best results with the serum, broth, and glucose mixture. Equal parts of 12% peptone and Dextraven gave the very high survival rate of 93%; moreover, the final product was better looking with the Dextraven plus peptone than with peptone alone.

It should be noted that although this Dextraven peptone mixture dried beautifully on the apparatus described in this paper, it was not satisfactory on the standard centrifugal drier; it frothed and finished as a scaly, glassy product.

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RESPIRATION OF MYCOBACTERIUM TUBERCULOSIS, STRAIN BCG, IN THE PRESENCE OF SHORT CHAIN FATTY ACIDS[1]

By E. Pascoe[2] and J. H. Quastel

Abstract

Rates of oxygen consumption by washed suspensions of *Mycobacterium tuberculosis*, var. *bovis*, strain BCG, in the presence of short chain fatty acids and other substrates have been studied. Whereas the presence of acetate, butyrate, and hexanoate leads to increased but constant rates of respiration, that of propionate, pentanoate, and heptanoate leads to increased rates of respiration that diminish markedly with time. Admixture of glucose or glycerol with short chain even-numbered fatty acids gives additive rates of oxygen consumption. Admixture, however, of glucose or glycerol with odd-numbered short chain fatty acids prevents the fall off in respiration and leads to increased and constant rates of oxygen utilization. These effects of glucose or glycerol are not due to the formation of fumarate or malate or of pyruvate or acetate. Mixtures of odd-numbered and even-numbered short chain fatty acids give rates of respiration which are (with the exception of acetate) those of the odd-numbered fatty acids alone. Acetoacetate and β-hydroxybutyrate resemble the short chain even-numbered fatty acids in this respect.

Introduction

Fat metabolism in mycobacteria has been a subject of considerable study, but details of the mechanism of breakdown and utilization of fatty acids in this group of microorganisms are still obscure. Only recently has it been shown (23) that fatty acids, at low concentrations, will support growth of mycobacteria. The optimal concentration for growth varies inversely with the molecular weight for fatty acids containing 12 or fewer carbon atoms.

It is well known (2, 4, 14, 23) that the presence of short chain fatty acids brings about an increased rate of oxygen consumption of mycobacteria. It has been our objective to secure further information concerning the effects of fatty acids on the respiratory activities of *Mycobacterium tuberculosis*, strain BCG. The purpose of the following paper is to present preliminary results obtained in an investigation of the metabolism of *M. tuberculosis*, strain BCG, studied in the form of washed intact cells, with special reference to the behavior of short chain fatty acids.

Methods and Materials

The organism used in these experiments was *Mycobacterium tuberculosis*, var. *bovis*, strain BCG. It will be designated by the abbreviation BCG in the remainder of this paper. Growth of the organism in Dubos Tween 80 albumin medium (5), after 12–16 days' incubation at 37° C., was harvested by centrifugation. The bacteria were then washed three times with, and finally suspended in, 0.1 *M* phosphate buffer, pH 7.3. The optical density of the

[1]*Manuscript received October 14, 1955.*
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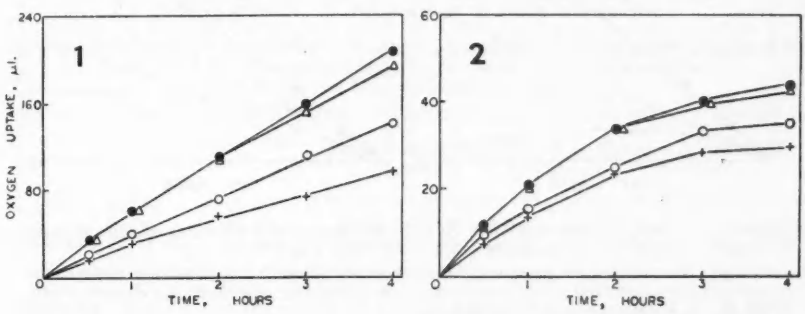
bacterial suspension was measured in a Fisher electrophotometer at 525 mμ and related to milligrams dry weight of bacteria per milliliter by reference to a standard curve drawn from the dry weights of previously prepared suspensions of known optical density.

Oxygen consumption was measured by the standard Warburg manometric technique. Neutral solutions of the substrates and phosphate buffer, pH 7.3 (final concentration 0.033 *M*), were placed in the main compartment of the vessel, the bacterial suspension (15 mgm. dry weight bacteria) in the side arm, and potassium hydroxide (0.2 ml. of 20%) in the center well. After the bacterial suspension was gassed with oxygen and equilibrated at 37° C., it was tipped into the main vessel, and manometric readings were taken for four hours.

Quantitative assays of glucose were made enzymatically by the use of notatin (12) and chemically by the copper–arsenomolybdate method (17, 19). After a four hour incubation period, the contents of the Warburg vessels were centrifuged and aliquots of the supernatant were assayed.

Oxygen Consumption of BCG in the Presence of Fatty Acids

Typical results showing the rates of oxygen consumption of BCG in the presence of short chain fatty acids containing even and odd numbers of carbon atoms are shown in Figs. 1 and 2 respectively. The results quoted have been corrected for the endogenous oxygen uptake which proceeds at a constant rate and whose magnitude is given in the legends to the figures. In the presence of butyrate (Fig. 1), BCG consumes oxygen at a constant rate. This rate varies directly with the concentration of the fatty acid until a saturation level is reached. Acetate and hexanoate exhibit similar results.



FIGS. 1 and 2. Effect of concentration of potassium butyrate (FIG. 1) and potassium pentanoate (FIG. 2) on oxygen consumption of BCG.

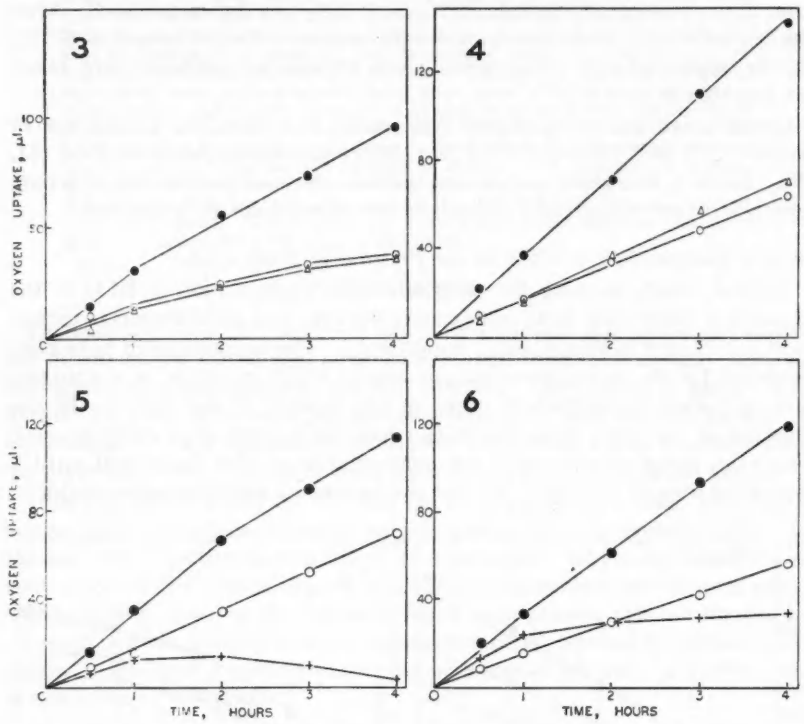
Vessel contents: 0.033 *M* phosphate buffer, pH 7.3; 15 mgm. dry wt. of bacteria in side arm; 0.2 ml. 20% KOH in center well; distilled water to 3.2 ml. Temperature, 37° C.; atmosphere, O_2; duration of experiment, four hours.

Results corrected for endogenous oxygen consumption (180 μl. in four hours).

FIG. 1. + K-butyrate, 0.005 *M*; ○ K-butyrate, 0.01 *M*; △ K-butyrate, 0.02 *M*; ● K-butyrate, 0.03 *M*.

FIG. 2. + K-pentanoate, 0.005 *M*; ○ K-pentanoate, 0.01 *M*; △ K-pentanoate, 0.02 *M*; ● K-pentanoate, 0.03 *M*.

The characteristics of oxygen consumption of BCG in the presence of odd-numbered fatty acids differ markedly from those in the presence of the even-numbered acids. Rates of oxygen consumption in the presence of pentanoate are shown in Fig. 2. The rates show a progressive diminution with time until, after two hours, no further oxygen is consumed. The final amount of oxygen consumed increases with increased fatty acid concentration until a maximum value is reached.



FIGS. 3-6. Oxygen consumption of BCG in the presence of mixtures of salts of fatty acids and glucose. Vessel contents as given for Figs. 1 and 2.

FIG. 3. Mixture used: butyrate + pentanoate. Results corrected for endogenous oxygen consumption (180 µl. in four hours). ● K-butyrate, 0.005 *M*; ○ K-pentanoate, 0.005 *M*; △ K-butyrate and K-pentanoate.

FIG. 4. Mixture used: butyrate + glucose. Results corrected for endogenous oxygen consumption (174 µl. in four hours). ○ glucose, 0.03 *M*; △ K-butyrate, 0.01 *M*; ● K-butyrate and glucose.

FIG. 5. Mixture used: propionate + glucose. Results corrected for endogenous oxygen consumption (175 µl. in four hours). ○ glucose, 0.03 *M*; + K-propionate, 0.01 *M*; ● K-propionate and glucose.

FIG. 6. Mixture used: pentanoate + glucose. Results corrected for endogenous oxygen consumption (138 µl. in four hours). ○ glucose, 0.03 *M*; + K-pentanoate, 0.01 *M*; ● K-pentanoate and glucose.

Similar characteristic curves of rates of oxygen consumption are obtained when BCG is incubated with propionate or heptanoate. At equimolar concentrations, propionate shows a lower, and heptanoate a higher, oxygen consumption than pentanoate. This is in accordance with the results of other workers (4, 6, 14).

Effects of Incubation of BCG with a Mixture of Even- and Odd-numbered Short Chain Fatty Acids

When BCG is incubated with a mixture of butyrate and pentanoate, each 0.01 *M*, the rate of oxygen consumed is not the sum of the separate rates, but is that of pentanoate alone. Typical results are shown in Fig. 3, where lower concentrations of fatty acid, 0.005 *M*, have been used. Typical values of oxygen consumption are shown in Table I, where the percentage of inhibition given is the average of the results of three or more experiments.

The same phenomenon is observed when pentanoate is replaced by propionate or heptanoate, and butyrate is replaced by hexanoate. With acetate, however, as substrate, additive rates of oxygen uptake are shown on admixture with odd-numbered fatty acids.

The fact that additive, or increased, rates of oxygen consumption are not obtained when the odd-numbered short chain fatty acids are added to the even-numbered fatty acids indicates that competition must take place, for the acids are used at concentrations well below those at which maximum velocities are observed (see Figs. 1 and 2).

TABLE I

EFFECTS OF ADDITION OF POTASSIUM PROPIONATE AND POTASSIUM PENTANOATE ON OXYGEN CONSUMPTION OF *Mycobacterium tuberculosis*, STRAIN BCG, IN FOUR HOURS AT 37° C. IN PRESENCE OF VARIOUS SUBSTRATES

(15 mgm. (dry weight) organism used in each experiment. Phosphate buffer present, 0.033 *M*, pH 7.3. Atmosphere, O_2. The values of oxygen consumption have been corrected for endogenous respiration)

		Propionate (0.01 *M*) present*		Pentanoate (0.01 *M*) present*	
Substrate (0.01 *M*)	μl. O_2 uptake	μl. O_2 uptake	Av. %† inhibition	μl. O_2 uptake	Av. %† inhibition
K-butyrate	76	39	49	13	76
K-hexanoate	226	116	48	96	65
Na-fumarate	30	14	53	—	—
Na-malate	37	4	91	—	—
K-acetoacetate	74	37	47	46	45
Na-β-hydroxybutyrate	86	30	61	43	45

* *The corrected O_2 uptake for K-propionate (0.01 M) alone = 9 μl. in four hours; that for K-pentanoate (0.01 M) alone = 25 μl. in four hours.*

† *Average for three experiments.*

Effects of Incubation of BCG with a Mixture of Odd-numbered Short Chain Fatty Acids and Acetoacetate or β-Hydroxybutyrate

The inhibitory effects of odd-numbered short chain fatty acids on the respiration of BCG in presence of even-numbered fatty acids are seen also when the latter are replaced by acetoacetate or β-hydroxybutyrate. Some typical experimental values are shown in Table I.

Effect of Incubation of BCG in the Presence of a Mixture of Glucose and Fatty Acids

Results given in Fig. 4 show the rates of oxygen consumption of BCG in the presence of glucose, butyrate, and a mixture of glucose and butyrate. The rate of oxygen consumption of BCG in the presence of the mixture is apparently equal to the sum of those in butyrate and glucose alone.

A similar additive result is obtained if acetate or hexanoate is substituted for butyrate.

The results differ markedly from those described, however, if a fatty acid containing an odd number of carbon atoms is substituted for butyrate. Results given in Fig. 5 show the rates of oxygen consumption of BCG in the presence of glucose and propionate, separately and together. In the presence of both glucose and propionate, the oxygen consumption is considerably in excess of the additive value. Typical experimental values are shown in Table II, where the percentages quoted are the average of three or more experiments. The rate of oxygen consumption for the mixture of glucose and propionate is constant throughout the experimental period. A similar result is obtained if propionate is replaced by pentanoate (Fig. 6) or heptanoate.

TABLE II

EFFECTS OF ADDITION OF POTASSIUM PROPIONATE AND POTASSIUM PENTANOATE ON OXYGEN CONSUMPTION OF *Mycobacterium tuberculosis*, STRAIN BCG, IN FOUR HOURS AT 37° C. IN PRESENCE OF VARIOUS SUBSTRATES

(15 mgm. (dry weight) organism used in each experiment. Phosphate buffer present, 0.033 *M*, pH 7.3. Atmosphere, O_2. The values of oxygen consumption have been corrected for endogenous respiration)

Substrate			Propionate (0.01 *M*) present*		Pentanoate (0.01 *M*) present*	
		μl. O_2 uptake	μl. O_2 uptake	Av. %† effect	μl. O_2 uptake	Av. %† effect
Glucose	0.03 *M*	70	128	+70	143	+54
Glycerol	0.01 *M*	77	146	+70	160	+60
Na-acetate	0.01 *M*	43	44	0	70	0
Na-pyruvate	0.01 *M*	24	23	0	—	—

* *See Table I for O_2 consumption of propionate and pentanoate alone.*
† *Average for three experiments.*

The rate of disappearance of glucose when this is incubated with BCG alone is not increased by admixture with propionate or pentanoate, in spite of the enhanced rate of oxygen consumption. For example, 20 μmoles glucose are consumed in four hours at 37° C. by 15 mgm. dry weight BCG in presence of 0.03 *M* glucose and 18.3 μmoles glucose are consumed under the same experimental conditions in presence of a mixture of 0.03 *M* glucose and 0.01 *M* potassium propionate.

The results are in harmony with the view that the utilization of odd-numbered short chain fatty acids in BCG is dependent on a chemical factor that is made available during the course of oxidative metabolism of glucose.

Effects of Incubation of BCG with a Mixture of Propionate and Glycerol

Glycerol has long been known as a source of carbon for the growth of tubercle bacilli (7, 8, 13, 21, 22) and as a substance that increases oxygen consumption (8, 9, 15, 16). In the presence of BCG and propionate, glycerol produces the same effect as glucose. Typical experimental values are shown in Table II. Glycerol, therefore, resembles glucose in creating conditions leading to constant rates of oxygen utilization in the presence of propionate.

Effects of Incubation of BCG with a Mixture of Propionate and Acetate or Pyruvate

Experimental values given in Table II show that neither acetate nor pyruvate behaves in a similar manner to glucose or glycerol. The results of additions of acetate or pyruvate to propionate are to produce rates of oxygen consumption that are approximately additive.

Effects of Incubation of BCG with a Mixture of Propionate and Fumarate or dl-Malate

Although fumarate and *dl*-malate are oxidized by *M. tuberculosis* var. *hominis* (6) and might be expected to be formed in the oxidative breakdown of glucose, their rates of oxygen consumption in the presence of BCG are considerably inhibited by the presence of propionate (see Table I for typical results).

Thus, it is evident that the action of glucose or of glycerol in enhancing the rate of oxygen consumption of BCG in the presence of odd-numbered short chain fatty acids cannot be due to the intermediate formation of fumarate or *dl*-malate.

Discussion

Details of the catabolism of fatty acids in mycobacteria are still unknown (6). While it may be anticipated that the breakdown of fatty acids in this group of organisms will follow the lines found in animal tissues and in other bacteria (3, 10, 20), attempts to isolate ketone bodies during fatty acid breakdown have been unsuccessful (14, 18). However, possible intermediates such as acetoacetate or β-hydroxybutyrate cause a relatively rapid rate of oxygen consumption in BCG and failure, therefore, to isolate such ketone bodies may be due to their rapid metabolism.

There are indications that the catabolic routes undergone by short chain fatty acids may differ from those of the longer chain members. Oginsky,

Smith, and Solotorowsky (18) noted that the oxidation of fatty acids containing 2 to 10 carbon atoms was unaffected by streptomycin, while that of the acids containing 12 to 18 carbon atoms was inhibited, and they have postulated different pathways of catabolism for the two groups.

The present manometric evidence shows that the odd- and even-numbered short chain fatty acids exhibit distinctive features in their effects on the respiration of mycobacteria. While the even-numbered fatty acids secure constant rates of oxygen uptake markedly higher than that of the endogenous, the odd-numbered fatty acids bring about an increased rate of oxygen consumption which falls off markedly with time. However, in the presence of glucose and glycerol (substrates which themselves bring about enhanced but constant rates of respiration), the odd-numbered fatty acids no longer exhibit diminishing rates of oxygen consumption. On the contrary, the mixtures show constant rates greater than those due to glucose or glycerol, similar to those brought about by the even-numbered fatty acids when present under the same experimental conditions.

The diminishing rate of oxygen consumption shown by the odd-numbered fatty acids, when added alone to mycobacteria, may be correlated with the fact that these substances depress markedly the increased respiration of mycobacteria when they are incubated with even-numbered short chain fatty acids or with substrates such as acetoacetate, β-hydroxybutyrate, fumarate, and *dl*-malate.

It is difficult to interpret these preliminary results on the basis of manometric evidence alone and clearly much further information derived from analytical studies and from the use of isotopes will be needed for an understanding of these phenomena.

It may be pointed out, however, that an inhibitory effect of propionate on butyrate metabolism in animal tissue has already been described (11) and it is reasonable to ascribe the inhibitory effects of odd-numbered short chain fatty acids to propionate formation following β-oxidation. It is likely (1) that propionate inhibition is due to propionyl-CoA production followed by competition between propionyl-CoA and acetyl-CoA but evidence for this, with mycobacteria, is, of course, still lacking.

It seems that glucose disappearance with mycobacteria is not affected by the presence of propionate or pentanoate, although an enhanced rate of oxygen consumption takes place. This may mean that glucose is more completely oxidized in the presence of propionate or pentanoate than in its absence, but it seems more probable that the oxidation of the odd-numbered short chain fatty acids in mycobacteria is stimulated by the presence of glucose and glycerol. Work on this problem is now in progress.

Acknowledgments

We are greatly indebted to the Institute of Microbiology of the University of Montreal for the strain of *M. tuberculosis* var. *bovis*, strain BCG, used in this work and to Dr. P. G. Scholefield of this Institute for the potassium salts of fatty acids used.

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A LYMPHOCYTOPENIA PRODUCING FACTOR FROM NOCARDIA[1]

By C. Margaret Charles Johnson, Joan de Vries, and J. W. Stevenson

Abstract

Nine strains of bacteria, isolated from the soil and provisionally classified as *Nocardia*, have been found to elicit a profound lymphocytopenia upon injection in heavy suspension into rabbits. Isolation of the haematologically active principle has been achieved by the tryptic digestion of whole cells, followed by centrifugation and prolonged dialysis of the supernatant fluid. The isolated material is of high potency, 1 mgm. producing in the rabbit a haematological response qualitatively identical to that obtained with whole cells and only slightly less in degree than that obtained with 100 mgm. of the lyophilized intact cells. On the basis of qualitative biochemical tests and paper chromatographic analysis, the active principle is believed to be a polysaccharide and was found to consist of reducing sugars and a non-reducing fraction, a "uronic" or "onic" acid, which gave the reactions characteristic of a sugar acid. The haematological activity and the chemical nature of the material suggests that this compound belongs to the large group of "pyrogenic" polysaccharides.

Introduction

During the course of an investigation of cholic acid metabolism, nine strains of bacteria, isolated by the soil perfusion method of Lees and Quastel (9), were shown by Halperin, Quastel, and Scholefield (7) to metabolize cholic acid actively producing as an end product a steroid, probably a 3-keto-Δ 4,5-cholanic acid derivative. All strains have been provisionally classified by the present authors as *Nocardia*. Further study of the organisms has shown that they induce a significantly consistent lymphocytopenia upon injection into rabbits. Because a steroid had been isolated as a product of the cholic acid metabolism and because the similarity of the lymphocyte pattern induced by these bacteria to that resulting from pituitary–adrenocortical stimulation indicated that an effect on the adrenal cortex might be the fundamental basis of the cellular response, the purpose of the present investigation was to isolate and attempt to make a preliminary characterization of the haematologically active principle.

Methods

The Test Organism

The identification of the test organism and its variants was carried out by the procedure followed by Jensen (8) in his classification of the "soil" corynebacteria, and they were tentatively classified according to *Bergey's Manual of Determinative Bacteriology* (1). All tests were performed on young, 24-hr. cultures obtained by subculturing into peptone broth from stock agar slopes. The stock cultures on agar slopes, originally obtained in the lyophilized state, were maintained by subculturing to fresh agar slopes every 14 days and storing at room temperature.

[1]*Manuscript received October 24, 1955.*
Contribution from the Department of Bacteriology and Immunology, McGill University, Montreal, Quebec. Aided by a grant from the National Research Council.

Morphology

Short and long, slightly curved, club-shaped rods; pleomorphic with aging, giving rise to distinct coccoid forms within 24 to 72 hr. No endospores. Not encapsulated. Non-motile. Gram-positive with many granules. Not acid-fast.

Culture Characteristics

Colonies on peptone agar, 2–6 mm. in diameter; low convex; entire edge with a tendency to confluence; salmon pink in young culture to orange pigment on aging; smooth and granular forms, glistening, opaque. Colonies on cholic acid agar smaller, 2–3 mm. in diameter; degree of pigmentation slightly more pronounced; other characters similar, with less tendency to confluence. Colonies on asparagine agar are similar to those on cholic acid agar, except the growth is somewhat more confluent; pigment initially pale salmon pink but after one to two weeks becomes a brilliant red. Growth in peptone broth finely granular with maximum growth at the surface and slight pellicle formation. Aerobic. Good growth at 25° C. and 37° C.

Physiological Characteristics

Heterotrophic. Free nitrogen not fixed. Gelatin not liquefied. Litmus milk, methylene blue, and malachite green milk unchanged. Nitrates rapidly reduced to nitrites, the maximum time being three days. H_2S not formed. Indole not produced. Cellulose not digested, starch not hydrolyzed. Voges–Proskauer test negative; urea positive. Catalase positive. pH of growth in glucose broth is 8.0. No acid or gas from glucose, sucrose, lactose, maltose, levulose, arabinose, xylose, galactose, mannose, trehalose, raffinose, inulin, dextrin, glycerol, mannitol, sorbitol, dulcitol, and salicin. Peptone water sugars were used. The only distinguishing characteristics between the nine strains are shown in Table I.

TABLE I

DIFFERENTIAL CHARACTERS OF THE *Nocardia* STRAINS

Species variant No.	Nitrite from nitrate		Sodium citrate	Sabouraud's media	Sodium hippurate broth	Asparagine agar
	1 day	3 days				
1	−	+	±	±	+	±
2	±	+	±	±	−	±
4*	±	+	+	±	−	±
5	−	+	+	±	±	±
7	±	+	+	+	+	+
10	−	+	+	+	+	+
11	±	±	±	±	−	++
12	+	+	+	+	±	±
13	±	+	+	+	−	+

**The test organism used throughout experiments. ±, slight growth in 48 hr.; +, good growth in 48 hr.; ++, heavy growth in 48 hr.; −, no growth in 48 hr.*

On the basis of their morphological, cultural, and physiological characteristics, all members of the group would seem to fall into the genus *Nocardia*. However, an identification such as this can only be regarded as tentative until the problem of the classification of the genera *Corynebacterium* and *Nocardia* has been satisfactorily settled (2).

Biological Assay of the Test Organism and Its Products

The test dose was suspended or dissolved, depending upon the nature of the material, in 1 ml. of sterile distilled water, or buffer at neutral pH (in the case of acid-material) and injected intramuscularly into rabbits weighing between 1.5 and 2.0 kgm. The animals were fasted 12 hr. prior to and throughout the experiment. Before the injection, 2 ml. of heparinized blood was taken from the marginal ear vein as a control, and 2-ml. samples were collected at the time intervals indicated below. The samples were used for differential blood counts and haematocrit determinations.

The blood for total leukocyte counts was taken directly from the ear vein. A minimum of 1000 cells were counted. The smears for differential counts were stained by the method of Speirs and Meyer (13). Five hundred cells were examined in each count and all results were expressed as the absolute values. The time necessary for the maximum response to occur was determined by taking five to six blood samples at two-hour intervals. To determine the duration of the response, two experiments were prolonged over an 11-hr. and a 32-hr. test period.

Control test doses employed were 100 mgm. of lyophilized bacterial cells, grown on a cholic acid fluid medium* and killed by heating at 60° C. for 30 min. on two successive days and 100 mgm. of lyophilized culture filtrate, obtained after centrifugation. As a test for the heat stability of the active principle, 100 mgm. of lyophilized bacteria, suspended in 1 ml. of sterile water, were autoclaved at 120° C. for 20 min. before injection.

Isolation of the Active Principle by Tryptic Digestion

Large scale culture of the test organism was carried out by a modification of the method designed by Reed and McKercher (12), involving culture on sheets of cellophane overlying filter pads soaked with peptone broth, and incubated at 37° C. for 48 hr. The harvested cells were washed twice with distilled water and lyophilized. The yield of lyophilized cells per 10 culture trays averaged 7.5 gm. Routine control tests for haematological activity of the whole cells were performed with lyophilized organisms in 25 mgm. and 50 mgm. doses suspended in 1.5 ml. distilled water and autoclaved at 120° C. for 20 min.

Proteolytic digestion of the cellular material was carried out according to the method reported by Nesset *et al.* (11). Five grams of whole cells were suspended in 1 liter of distilled water and autoclaved at 120° C. for 20 min.

K_2HPO_4, 0.5 gm.; $MgSO_4 . 7H_2O$, 0.2 gm.; $NaCl$, 0.2 gm.; $MnSO_4 . 4H_2O$, trace; $FeCl_2 . 6H_2O$, trace; NH_4Cl, 0.5 gm.; ammonium salt of cholic acid, 1.0 gm.; distilled H_2O, 1000 ml.; pH adjusted to 7.0; sterilized by Seitz filtration.

The suspension was adjusted to pH 8.5 with 2 *N* NaOH, layered with toluene, and incubated at 37° C. for five days. A 50 mgm. portion of trypsin (Nutritional Biochemicals Corp.) was added initially and another equal portion was added after two and one-half days, to make the enzyme–substrate ratio 1 : 50. The pH was maintained between 8.0 and 8.5 by additions of sodium hydroxide.

Following the five day incubation period, the digest was centrifuged at 2000 r.p.m. for one hour to remove the cellular debris. The supernatant was transferred to cellophane tubes (dialysis tubing, Fischer, 0.00072 in., width 1⅝ in.) and dialyzed against running tap water for 48 hr., during which time the tubes were occasionally inverted to ensure complete dialysis. The residual content of the cellophane tubes was centrifuged at 2000 r.p.m. for two hours, the supernatant concentrated *in vacuo*, and lyophilized. This material, designated "tryptic concentrate" was tested for haematological activity. Characterization was carried out by the usual biochemical methods, and the biological activity of the material was investigated.

Results

Haematological Activity of the Whole Cells and Culture Filtrate

It was established that the various culture products of the soil bacteria grown on a cholic acid fluid medium elicited a consistent and marked haematological response. Within a period of three and one-half hours following injection the numbers of lymphocytes in the circulating blood stream dropped some 52 to 72%. The lymphocytopenia was usually accompanied by a drop in the total white cell count; however, this was not consistent, a leukocytosis being found in some cases. A marked neutrophilia was also found to occur. Monocytes and basophiles, although routinely counted, showed no significant change. Figs. 1 and 2, and Tables II, III, and IV show

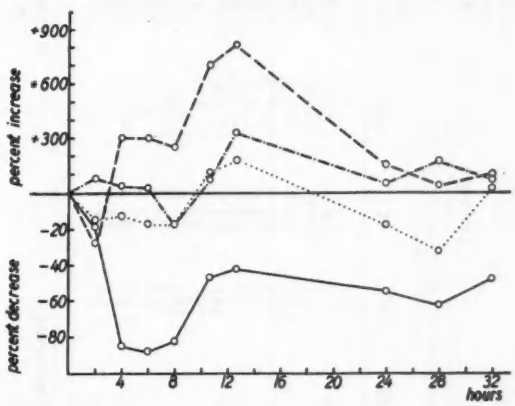

FIG. 1. The haematological response to lyophilized heat-killed cells—100 mgm. ····· Total white blood cells; ———— small lymphocytes; — — — — — neutrophiles; —·—·—·— eosinophiles.

TABLE II

CONTROL: THE HAEMATOLOGICAL RESPONSE TO LYOPHILIZED HEAT KILLED CELLS—100 MGM.

Rabbit	Hr.	Ht.*	% change	Small lymphocytes	% change	Neutrophiles	% change	Eosinophiles	% change	Total W.B.C.	% change
1818	0	46.0	0	10,086	0	2580	0	88	0	14,660	0
	1¾	No ht.	No ht.	8177	−18.9	1836	− 28.8	171	+ 94.3	12,242	− 16.4
	3¾	45.8	− 0.4	1577	−84.2	10,265	+297.8	128	+ 45.4	12,800	− 12.6
	5¾	44.0	− 4.3	1295	−87.1	12,050	+297.2	100	+ 13.6	12,450	− 15.0
	7¾	44.0	− 4.3	1928	−80.8	9517	+268.8	74	− 15.9	12,360	− 15.6
	11¼	43.5	− 5.6	5400	−46.4	20,580	+697.6	122	+ 38.6	30,720	+109.5
	13¼	43.4	− 5.6	5750	−42.9	23,959	+828.6	343	+289.7	34,325	+134.0
	24¼	43.4	− 5.6	4475	−55.6	6620	+156.5	144	+ 63.6	12,035	− 17.9
	28½	39.8	−13.4	3770	−62.6	3950	+ 53.1	236	+168.1	9835	− 32.9
	31¾	37.5	−18.4	5150	−48.9	6202	+140.3	163	+ 85.2	13,580	+ 7.3
1831	0	41.4	0	4770	0	2360	0	40	0	9910	0
	5½	40.0	− 3.3	750	−84.2	4070	+ 72.4	0	−100.0	5135	− 48.2

**Haematocrit.*

TABLE III

CONTROL: THE HAEMATOLOGICAL RESPONSE TO LYOPHILIZED CULTURE FILTRATE—100 MGM.

Rabbit	Hr.	Ht.	% change	Small lymphocytes	% change	Neutrophiles	% change	Eosinophiles	% change	Total W.B.C.	% change
1816	0	42.7	0	9150	0	3090	0	136	0	13,675	0
	1¾	42.0	−0.6	10,080	+ 4.8	6640	+114.8	222	+ 63.2	18,650	+36.3
	3¾	42.5	−0.4	2511	−72.5	5359	+ 73.4	85	− 37.5	8485	−37.9
	5¾	41.0	−3.9	3529	−61.4	12,966	+319.6	0	−100.0	17,475	+27.7
	7¾	No ht.	No ht.	2350	−74.3	5120	+ 65.6	17	− 87.5	8501	−37.8
	11	40.0	−6.3	1830	−80.0	10,755	+248.0	56	− 58.8	13,860	+ 1.3
1832	0	No ht.	No ht.	10,640	0	1075	0	55	0	13,770	0
	5½	40.0	No ht.	2610	−75.4	9010	+764.1	134	+143.6	13,450	− 2.3

TABLE IV

THE HAEMATOLOGICAL RESPONSE TO LYOPHILIZED BACTERIAL CELLS AUTOCLAVED AT 120° C. FOR 20 MIN.—100 MGM.

Rabbit	Hr.	Ht.	% change	Small lymphocytes	% change	Neutrophiles	% change	Eosinophiles	% change	Total W.B.C.	% change
1831	0	41.4	0	4770	0	2360	0	40	0	9910	0
	5½	40.0	−3.3	750	−84.0	4070	+72.04	0	−100	5135	−48.1

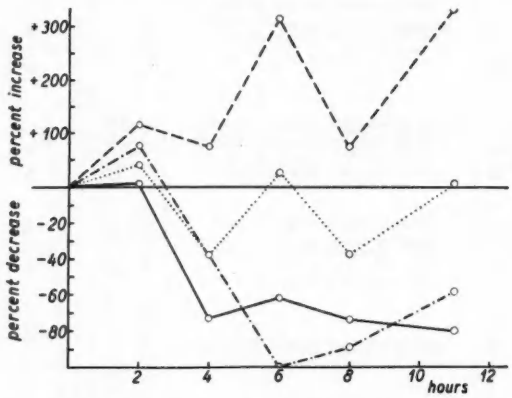

FIG. 2. The haematological response to lyophilized culture filtrate—100 mgm. ····· Total white blood cells; ———— small lymphocytes; — — — — — neutrophiles; —·—·—·—· eosinophiles.

the results obtained which served as controls for the activity of the test organism. That cholic acid and other constituents of the medium contributed nothing to the response was confirmed. In three experiments involving the injection of lyophilized, sterile cholic acid medium there was a slight increase in lymphocytes, neutrophiles, and total count, a situation quite the opposite to that seen when culture products were used. In addition, the characteristic white cell response was found not to be limited to those organisms grown in a cholic acid medium; equally good results were obtained with organisms grown in peptone broth and on peptone agar. For this reason, it was possible to use the large-scale culture technique described in the preceding section.

The control tests for the haemotological activity of the whole autoclaved bacterial cells, which had been harvested from peptone broth culture, were positive when a 50 mgm. dose was given but negative in 25 mgm. quantities. The amount of the active principle in 25 mgm. of cells was considered to be inadequate for the production of a significant response (Table V).

In order to adopt this characteristic haematological response as a method of following the progress of isolation of the active principle, it was necessary to standardize several conditions. On the basis of the results obtained over an eight-hour test period, it was found that the maximum response was consistently bracketed within three and one-half to four hours. Because the normal white cell counts and cellular proportions in rabbits normally vary to a considerable extent, some standard criterion as to the degree of response which could be regarded as significant had to be established. The average values and their Standard Deviations were determined in an analysis of all the normal count determination made in some 45 experiments. In each experiment, as has been noted in a previous section, a minimum of 1000 cells were counted to determine total counts; in the differential counts 500 cells

TABLE V

THE HAEMATOLOGICAL RESPONSE TO WHOLE LYOPHILIZED BACTERIAL CELLS—(*a*) 25 MGM., (*b*) 50 MGM.

Rabbit	Hr.	Ht.	% change	Small lymphocytes	% change	Neutrophiles	% change	Eosinophiles	% change	Total W.B.C.	% change
1833	0	44.1	0	7170	0	1500	0	394	0	13,160	0
(*a*)	4	44.0	0	6940	− 3.2	3810	+ 154.0	142	−63.9	14,210	+7.9
1844	0	45.0	0	19,250	0	1845	0	154	0	25,600	0
(*b*)	4	43.1	−4.2	10,761	−44.0	13,110	+1010.5	214	−38.9	26,770	+4.5

TABLE VI

THE STANDARD DEVIATION OF NORMAL WHITE CELL COUNTS IN 45 EXPERIMENTS, TOTAL NUMBER/CU.MM.

Cells	Absolute numbers	S.D. %
W.B.C.	15,082 ± 2319 (S.D.)	15.4
Small lymphocytes	8636 ± 3107 (S.D.)	35.8
Neutrophiles	3685 ± 2151 (S.D.)	55.13
Eosinophiles	133 ± 101 (S.D.)	76

were examined and the results expressed as absolute values on the basis of the total count. An analysis of average values and their standard deviations is presented in Table VI.

On the basis of the data, *a decrease* in lymphocytes of 36% or more was considered significant. An *increase* in neutrophiles of 55% or more, although of secondary importance in the analysis of the response, was also statistically significant. The standard deviation obtained for eosinophiles would seem to indicate that no real statistical significance may be attached to the response of that cell, presumably because of the extremely wide variations among the rabbits and the small numbers counted.

Isolation and Preliminary Characterization of the Active Lymphocytopenic Principle

In order to remove practically all traces of protein material after tryptic digestion, it was found that a period of at least 48 hr. or more was required, as opposed to the 24 hr. period suggested by Nesset *et al.* (11). The yield of concentrate was 891.6 mgm. per 5 gm. of dried cells, or 17.83%. The material was light buff in color and of a spongy consistency and felt similar to starch in texture. It was insoluble in water but formed a fine colloidal suspension, and could be easily handled. It was found to be soluble in a weak solution of calcium hydroxide.

The following protein tests on an 0.2% distilled water suspension were negative; biuret, ninhydrin, Hopkins–Cole, xanthoproteic, Millon, Sakaguchi, sulphur, and heat coagulation. The Molisch test for polysaccharide was positive. Micro-Kjeldahl analysis showed that the concentrate contained 0.18% nitrogen as compared to 4.9% nitrogen in the whole cells. After hydrolysis, by refluxing 200 mgm. of concentrate with 30 ml. of 1 *N* HCl for five hours, and neutralizing to pH 6.8 with 10 *N* NaOH, the Benedict and Barfoed tests for reducing sugar, and the Dische reaction for glucuronic acid or polyglucuronides (3) were positive. The tests for glucosamine and chondrosamine (4), and N-acetyl-glucosamine and N-acetyl-chrondosamine (10) gave a very faint red color which was considered doubtful and non-interpretable. The benzidine (Tauber) test for pentoses and the nitric acid test for purine bases and derivatives were negative. Spectrophotometric analysis of this

material in dilute calcium hydroxide showed maximum absorption to be at 210 mμ with a very small peak resulting. It was noted that an absorption peak in the region characteristic of the steroid mentioned in the introduction (240 mμ in alcohol) was not revealed.

Through the courtesy of Dr. S. Baker of the Montreal General Hospital Research Institute, an attempt was made to characterize the hydrolyzed tryptic concentrate by means of paper chromatography. Because of the salts present in the hydrolyzate and the small amount of material which was available at the time, only an initial characterization could be made.

The chromatograms were developed for amino acids using the ninhydrin reagent and for aldoses or reducing sugars with aniline hydrogen phthalate reagent. The presence of a small concentration of amino acids was detected by this method. Reducing sugars were present in a somewhat larger concentration, but hexosamine specifically was not demonstrated. The largest and most concentrated spot which was demonstrated did not develop with either ninhydrin or aniline hydrogen phthalate. It was felt that this spot represented the major constituent of the haematologically active compound.

Since it was believed that a non-reducing sugar acid or its salt was responsible for the large unidentified spot, it was cut out from the rest of the chromatogram, and the naphthoresorcinol (Tollen) test for glucuronates was applied. The filter paper containing the non-reducing substance was suspended in water and the test was performed before the paper was removed. The reaction was positive.

The haematological responses to both 1 mgm. and 0.1 mgm. doses of the concentrate were strongly positive, indicating a high degree of concentration of the material. The graphical representation illustrates that the character of the response is identical to that obtained when whole cells or filtrate were injected (Table VII and Fig. 3).

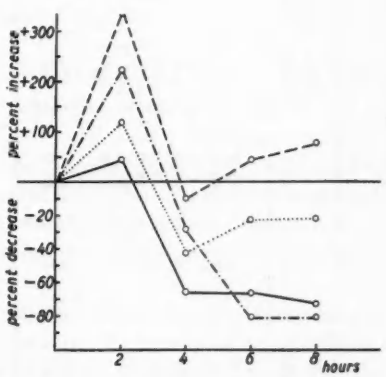

FIG. 3. The haematological response to the protein-free tryptic concentrate—1 mgm. ····· Total white blood cells; ———— small lymphocytes; — — — — — neutrophiles; —·—·—·— eosinophiles.

TABLE VII

THE HAEMATOLOGICAL RESPONSE TO THE PROTEIN-FREE TRYPTIC CONCENTRATE—(*a*) 1 MGM., (*b*) 1 MGM. (RESULTS PLOTTED IN GRAPH), (*c*) 0.1 MGM.

Rabbit	Hr.	Ht.	% change	Small lymphocytes	% change	Neutrophiles	% change	Eosinophiles	% change	Total W.B.C.	% change
1851	0	41.0	0	7630	0	9100	0	81	0	20,310	0
(*a*)	4	39.0	− 4.8	3200	−58.0	8281	− 9.0	0	−100.0	12,900	− 36.4
1884	0	42.8	0	9570	0	5550	0	141	0	17,580	0
(*b*)	2	39.0	− 8.8	14,100	+47.3	20,000	+350.3	460	+226.2	38,460	+118.7
	4	38.0	−11.2	3260	−65.9	5000	− 9.9	100	− 29.0	10,000	− 43.1
	6	38.0	−11.2	3080	−67.8	8050	− 45.0	27	− 80.8	13,400	− 23.7
	8	37.0	−13.5	2510	−73.7	9450	+ 70.2	27	− 80.8	13,670	− 22.2
1853	0	46.6	0	5890	0	3620	0	299	0	11,520	0
(*c*)	4	46.7	0	1670	−71.6	8180	+125.9	137	− 54.1	11,430	− 0.7

In summary, the carbohydrate nature of the active principle has been established. An evaluation of the method of proteolysis of dried cells with trypsin shows it to be quite satisfactory. The concentrate which is produced, although insoluble is in a form which is readily handled and analyzed. However, whether all of the constituents of the material have been detected has not been determined. Furthermore, because analysis must be performed on hydrolyzed material, no knowledge beyond hypothesis has been gained as to the constituents necessary for biological activity.

Discussion

Because a steroid had been isolated as an end product of cholic acid metabolism by the soil organisms, and because there was a close similarity of the haematological response elicited by the cells to that of ACTH and cortisone, it had been suggested that the steroid might be responsible for the biological activity. This does not seem to be the case, primarily because the spectrophotometric analysis of the biologically active fraction did not reveal an absorption peak characteristic for the steroid (240 mμ in alcohol). Furthermore, because the organisms grown in peptone broth also elicited an identical response it is apparent that the active property of the cells was not dependent on the metabolism of cholic acid as such. However, investigations as to whether the same steroid is present in the cells when grown in broth have not been carried out.

Of the isolation procedures attempted, tryptic digestion of the whole cell suspension with centrifugation and prolonged dialysis, as reported in this paper, gave the most satisfactory results. The concentrated material was obtained in satisfactory yield and consisted almost exclusively of carbohydrate; it would appear from the analysis that the molecule responsible for the biological activity is a complex carbohydrate as a polysaccharide. However, the individual constituents of the complex have not been determined with certainty.

It is tentatively proposed on the basis of the evidence presented that the active principle is composed of reducing sugars and a non-reducing fraction, possibly a "uronic" or "onic" acid. That the small trace of protein derivatives detected in the concentrate is not a component of the active molecule cannot be ruled out with certainty, although there is some evidence that its presence is not essential to haematological activity, and is due to residual contamination. In this repect, further investigation with the absolute removal of protein would be necessary.

The biological, physical, and chemical properties would seem to relegate the active concentrate to the group of so-called bacterial "pyrogens" (6). Moreover, the results of our analyses compare favorably with the results of analyses of bacterial pyrogens made by Nesset *et al.* (11) and by Ginger *et al.* (5). Indeed, the very fact that successful isolation of the haematologically

active principle was achieved by a slight modification of the isolation methods of those workers would indicate that the two compounds might be of a closely similar nature.

The haematological response of the rabbit induced by the bacterial cells, culture filtrates, and the concentrated cell derivative has proved to be quite reliable. The toxicity of the active principle, cells, and filtrates, when given in single test doses, does not seem to be of any significance. The rabbits showed no symptoms of any toxic effects and the white blood cell count returned to normal within a period of several days.

We feel that the results of this fractionation procedure followed by the haematological response compare favorably to those obtained by workers employing the usual method of assay, i.e. the measurement of the degree of febrile response induced by intravenous injection into the rabbit. The sensitivity of the rabbit, as the test animal, to doses of the purified material in the range of 0.1 mgm. indicated that the haematological response shows a high degree of specificity. Although it is not our purpose to propose that this method might be more advantageous, it should be pointed out that, in our investigations and in those experiments using the fever assay method, compounds of a similar nature have been successfully isolated.

Acknowledgment

The authors gratefully acknowledge the technical assistance of Mrs. Ruth Lamartine Sprott.

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THE USE OF DUBOS OLEIC ACID ALBUMIN MEDIUM ENRICHED WITH BLOOD FOR THE VIABLE COUNTING OF BCG[1]

By Doraine C. W. Thow[2]

Abstract

Several different media were compared by means of surface viable counts of suspensions, in 0.1% solution of bovine serum albumin, of freshly grown BCG, and of BCG resuspended after freeze-drying. The growth of BCG on oleic acid albumin medium enriched with 5% whole blood was best in three respects: from a given inoculum, the colony count was highest; the colonies became visible soonest; and reached the greatest maximum size. The superiority of this medium was particularly evident with inocula of BCG reconstituted after freeze-drying.

Introduction

Examination of the literature reveals little, if any, agreement among European and North American investigators about the percentage survival of organisms in BCG vaccines after freeze-drying. This is hardly surprising, because in each investigation not only a different method and medium for counting the viable organisms were used, but also a different method of drying. The possibility that the number of colonies growing from a suspension of viable organisms might vary with the type of medium employed, especially after freeze-drying, does not seem to have been considered by any investigators, who all used only one routine medium for their viable counts (1, 2, 3, 4, 12, 16, 18, 20, 23, 24, 25, 28, 29, 30).

Early in the investigations reported in this paper it became clear that colony counts from standard inoculations made both before and after freeze-drying of the organisms, varied substantially with the medium. This variation was obviously important in counting reconstituted freeze-dried suspensions; some media supported no visible growth and others a much heavier growth at the same dilution. For this reason, special attention was given to the development of a medium suitable for viable counts of suspensions of BCG.

Methods

Standard inocula of suspensions containing relatively small numbers of well-dispersed organisms constitute a most sensitive means of evaluating the growth-initiating and growth-supporting properties of any medium. The clear, semisynthetic, oleic acid albumin medium described by Dubos and Middlebrook (9) was used as a standard for the following reasons. It supports the growth of small numbers of tubercle bacilli; it is easily made;

[1]*Manuscript received November 29, 1955.*
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and because it is semisynthetic it varies little in successive batches, unlike egg media which vary unpredictably both from batch to batch and from plate to plate in the same batch. Unstained egg media also have the great disadvantage that owing to their creamy yellow color, discrete colonies are not easily visible, especially in young cultures, and so may be missed in the colony count.

The Lowenstein–Jensen medium used in these experiments was made routinely in the Department as follows. Solution I: KH_2PO_4, 0.9 gm.; $MgSO_4$, 0.09 gm.; Mg citrate, 0.225 gm.; asparagine, 1.35 gm.; glycerol, 4.5 ml.; distilled water, 225 ml. Dissolve in steamer at 100° C. for two hours. Leave in the refrigerator at 4° C. overnight. To 225 ml. of Solution I add 11.5 gm. of potato starch. Heat this to 70° C. until a smooth paste is obtained and then place in a 56° C. water bath for one hour. Solution II: Add 375 ml. of shelled eggs, well-mixed, to Solution I, then add 7.5 ml. of a 2% solution of malachite green. Filter through muslin. Fill the plates with this medium and coagulate it by heating at 70° C. in an inspissator for one hour on three successive days.

With this medium results often proved unpredictable; with one batch of medium the viable counts might be very high and with others very low. In some cases plates showed no growth after six weeks' incubation. Occasionally, for no apparent reason, there were one or two plates in a batch which either showed no growth or a single colony where confluent growth was to be expected. A further disadvantage of Lowenstein–Jensen medium is the amount of care necessary for its successful manufacture if it is to yield good cultures. This point was stressed by Jensen (15).

The strains used were obtained from Dr. J. Bøe at the BCG Laboratory in Bergen. They were maintained in Dubos Tween 80 albumin medium by subculture at intervals of two to three weeks. In this medium, Dubos, Fenner, and Pierce (8) maintained standard cultures of mycobacteria by weekly passage for some three years, during which period the organisms retained their virulence and morphological characters.

Cultures of BCG in this medium became turbid in about five days, the growth consisting of microscopic clumps. After 14 to 21 days there was a heavy white deposit of growth on the bottom of the flask, which on shaking dispersed into a uniformly turbid suspension, free from macroscopic clumps. Prolonged or violent shaking produced small visible clumps.

Preparation of the Suspension of BCG

The method was modified from Fenner, Martin, and Pierce (13). A two-week-old culture of BCG in Dubos Tween 80 albumin medium was shaken in a Kahn shaker for 15 min. to disperse the growth as much as possible, and filtered through two sheets of Whatman No. 2 filter paper to remove the larger clumps. The clear filtrate contained approximately 53% single-cell, 11% double-, 2% triple-, and 1% quadruple-cell particles, the remaining 33% being

clumps of five cells or more. This suspension was concentrated twofold by centrifugation, and then contained about two to five million viable particles per milliliter.

Viable Counts

The method was a modification of that of Miles and Misra (21). Serial 10-fold dilutions (0.5 ml. + 4.5 ml. diluent) of the suspension were made in 0.1% bovine serum albumin in distilled water, using a fresh pipette for each dilution (31). Petri dishes of media, agar surface up, were inoculated by means of calibrated dropping pipettes (6). Five 0.02 ml. drops of the highest dilution of suspension were seeded on five marked areas on each of three plates of a given medium. The dropping pipette was then rinsed and used for the next highest dilution, and the procedure repeated for each of the lower dilutions. For a given medium, therefore, each dilution was represented by a total of 15 drops distributed on three plates. As a rule, three media were seeded simultaneously in parallel, so that nine plates were used for each dilution. When the free fluid of the inoculum had been absorbed, the plates were sealed with "Sellotape", and incubated at 37° C.

The area of medium covered by each drop was 10–12 mm. Since from five to seven 10-fold dilutions of suspensions were seeded, it will be obvious that in drop areas from the lower dilutions, growth was confluent. In practice, usually only two suspensions, differing in concentration by a factor of 10, yielded countable numbers of colonies in the drop areas. In the tables, counts from one or two of these dilutions only are cited.

Readings were made at intervals and the maximum colony count recorded. The highest mean drop-count in any series was used as an index of the value of the medium regardless of the period of incubation taken to reach the maximum. With further incubation, counts declined from the maximum, owing to increasing confluence of the colonies. With large numbers of colonies per drop-area the decline was reached more quickly than with small numbers on the same medium. Counts above a mean value of 30 often proved inaccurate owing to the semiconfluence of the growth. When colonies were

TABLE I

COMPARISON OF DROP-COUNTS ON TWO MEDIA

Dilution	Plate No.	OAA medium					Mean	OAA + 5% laked blood medium					Mean
		Colonies per drop						Colonies per drop					
5×10^{-4}	1	11	16	23	11	9	14.0	34	29	41	48	40	38.4
	2	15	7	6	5	9	10.4	43	41	28	43	37	38.4
	3	11	13	15	14	14	13.4	40	31	30	27	50	35.6
	Total						12.6						37.5
5×10^{-4}	1	2	1	3	1	0	1.4	3	6	2	5	3	3.8
	2	2	1	1	0	0	0.8	2	3	2	2	2	2.4
	3	2	1	2	1	0	1.2	4	3	3	6	2	3.6
	Total						1.1						3.3

well isolated, usually no increase in the count was observed; but even well-isolated colonies became confluent when the growth was vigorous and spreading, as occurred from time to time, especially on enriched media.

Table I records a typical count on two different media and shows the consistency of the counts from drop to drop and plate to plate for a given dilution. The mean of each set of 15 values was determined in all the tests, and represents the mean number of viable particles per 0.02 ml. of dilution seeded.

Preparation of the Suspension of BCG for Freeze-drying

Equal amounts of the suspension were thoroughly mixed with a fluid menstruum (e.g. serum, glucose and serum, or 50% glucose); 0.25 ml. or 0.1 ml. amounts were freeze-dried in test tubes 10 × 0.8 cm. and sealed under vacuum. The dried suspensions were reconstituted in 5 ml. of 0.1% bovine serum albumin, corresponding to a 5×10^{-2} or 2×10^{-2} dilution of the original material, depending on the amount dried.

Results

Two types of medium were tested: inspissated egg media containing various adjuvants; and semisynthetic media containing various mineral salts, digests, proteins, amino acids, and agar.

The Lowenstein–Jensen medium proved to be slightly better than OAA medium. Counts on Lowenstein–Jensen medium were higher in 48%, equal in 17%, and lower in 35% of the tests made.

King and Lucas (17) described a bean–egg medium which they found to be better than Dorset's, Petroff's, Corper and Cohn's, and slightly better than Petragnani's egg media. Table II summarizes a comparison of Petragnani's (19), King and Lucas's, and Lowenstein–Jensen medium with and without glycerin. The results are rather conflicting. The unexpectedly high counts obtained on the Lowenstein–Jensen medium, with or without glycerin, at 10^{-4}, together with a count of 90 at 10^{-3}, shows that the suspension of organisms must have contained at least five million bacterial units per milliliter. Assuming that this is so, Petragnani's medium and King and Lucas's medium both fall far short of indicating the maximum number of viable organisms.

TABLE II

MEAN DROP-COUNTS ON FOUR EGG MEDIA

Series	Dilution	Petragnani's medium	King and Lucas's medium	L-J medium	L-J medium without glycerin
1	10^{-3}	39.8	C	90.0	21.6
	10^{-4}	4.4	1.8	2.0	3.6
2	10^{-3}	39.0	28.2	42.6	38.2
	10^{-4}	5.0	5.2	17.2	17.8

L-J = *Lowenstein–Jensen.*
C = *Contaminated plates.*

TABLE III

RATES OF GROWTH ON FOUR MEDIA

Period of incubation (weeks)	Dilution	OAA medium	L–J medium	L–J medium + 5% glucose	OAA medium + 15% egg yolk
2	10^{-3}	**33.4**	+	++	0.0
	10^{-4}	2.8	0.0	0.0	0.0
	10^{-5}	0.0	0.0	0.0	0.0
3	10^{-3}	+++	19.2	40.5	+++
	10^{-4}	**5.4**	1.9	5.9	9.0
	10^{-5}	0.2	0.0	**0.6**	**1.6**
4	10^{-3}	+++	**21.2**	**44.8**	+++
	10^{-4}	5.4	2.9	6.6	**10.2**
	10^{-5}	**0.4**	0.0	0.6	1.6

L–J = Lowenstein–Jensen.
Earliest maximum counts are indicated in heavy type.

Investigators (5, 10, 14, 26) have reported that media composed either wholly of egg yolk or enriched by it yielded more growth than other kinds, including media made of whole egg. The growth stimulating effect of egg yolk was tested in OAA medium; 15% (v/v) was used. The counts were anomalous. With freshly grown bacilli, the drop counts on OAA in 10^{-4} was 5.4, and on OAA with egg 10.2. An opposite result, however, was obtained with a reconstituted suspension; a dilution yielding 27.8 on OAA entirely failed to grow on OAA plus egg. Because of the difficulty of seeing colonies against the pale yellow background of this OAA-egg medium, and of the ease with which it became contaminated, malachite green, 1 : 10,000, was added as a bacteriostatic and as a stain for the background. The dye, however, completely inhibited the growth of BCG. Moreover, as the last column of Table III shows, growth is slower on the yolk-enriched medium. For these reasons egg-yolk enrichment was not investigated further.

Nassau (22) found that the addition of 1% blood charcoal to solid or liquid media increased the rate of growth of, and the numbers of colonies developing from a human strain of the tubercle bacillus. The addition of a 1% suspension of activated blood charcoal to the OAA medium improved growth in one test, but made no difference in another. There was no increase in the speed of growth.

An autoclaved 1% solution of water laked horse blood improved the growth on OAA medium on the two occasions it was tested. A 1% solution of pure haemoglobin increased the growth in one experiment, but apparently lacked something present in the laked blood because in two other tests the count was lower than on plain OAA.

Tarshis and Frisch (27) demonstrated that agar media containing 15–50% human blood supports the growth of small inocula of tubercle bacilli. Neither the age of the blood nor its citrate content affected its growth promoting

TABLE IV

EFFECT OF VARYING THE BLOOD ENRICHMENT OF OAA MEDIUM

Experiment No.	Dilution	OAA medium + 5% laked blood	OAA medium + 10% laked blood	OAA medium + 25% whole blood
1	2×10^{-3}	45.0	42.0	44.0
	2×10^{-4}	8.0	1.8	5.0
2	2×10^{-3}	18.8	—	12.0
	2×10^{-4}	3.0	—	2.3
3	2×10^{-3}	49.6	56.1	57.6
	2×10^{-4}	5.4	5.0	4.7
4	2×10^{-3}	40.8	37.2	42.7
	2×10^{-4}	3.4	3.3	4.1
5	2×10^{-3}	27.0	20.8	20.7
	2×10^{-4}	2.0	2.0	—
6	2×10^{-3}	19.9	18.4	18.0
	2×10^{-4}	2.3	2.6	2.9

powers; and they therefore recommended the use of discarded bank blood. A suspension yielding a count of 31.5 on OAA failed to grow on trypsin agar (a tryptic digest of horse muscle plus 2% agar final concentration) or trypsin agar with 5% whole human blood; but the count was 30 on trypsin agar with 25% whole blood. The 25% blood agar medium, however, was inferior to the OAA medium in that it failed to support the growth of smaller numbers of organisms.

The good effect of blood enrichment with trypsin agar suggested a trial of blood-enriched OAA medium. Saponin-laked and whole blood were compared, using reconstituted suspensions of BCG. There was no significant difference in the growth obtained with 5% and 10% laked blood and 25% whole blood (Table IV).

Compared with OAA alone, OAA plus 5% laked blood was a slightly better medium for freshly grown bacilli. In 4 of 11 separate tests, the counts were about equal, but in the remaining seven, as the ratios in the last column of Table V show, the blood enriched medium was better, by a factor ranging from 1.15 to 3.15.

With reconstituted freeze-dried bacilli, however, 5% laked blood definitely improved the OAA medium in all of 14 separate tests made in the same way as the 11 tests recorded in Table V. The ratio of the counts, enriched: unenriched medium, were, in order of magnitude, 2.5, 2.5, >2.5, 2.8, 3.0, 3.0, 3.5, 4.0, >6.5, 10.5, 13.0, >14.5, >21.5, >36.0.

Eleven similar comparisons were made of 5% laked and 5% whole blood in OAA medium. The ratio of the 11 pairs of counts, laked: whole, in order of magnitude were, 0.8, 1.0, 1.1, 1.1, 1.2, 1.3, 1.3, 1.5, 1.5, 1.6, 1.7. That is, the whole blood is slightly better than the laked.

TABLE V

EFFECT OF BLOOD ENRICHMENT OF OAA MEDIUM

Experiment No.	Dilution	OAA medium (*a*)	OAA medium 5% laked blood (*b*)	Ratio (*b*/*a*)
1	10^{-4}	5.0	6.7	1.3
2	10^{-3}	14.8	20.4	1.35
3	10^{-3}	16.9	23.1	1.35
4	10^{-2}	30.8	45.2	1.5
	10^{-3}	3.1	5.3	1.7
5	10^{-3}	23.3	23.2	1.0
6	10^{-4}	6.3	10.5	1.7
7	10^{-2}	9.0	15.5	1.7
8	10^{-3}	40.3	46.8	1.15
	10^{-4}	4.4	4.4	1.0
9	10^{-3}	38.8	39.5	1.0
	10^{-4}	3.7	4.3	1.15
10	10^{-3}	9.2	29.1	3.15
11	10^{-4}	33.0	34.0	1.0

These experiments show, then, that there is a substantial increase in the proportion of bacilli in living suspensions which develop into colonies when media are enriched with blood, and that whole blood is a slightly better initiator of growth than laked blood. The greatest effect of blood enrichment was seen when freeze-dried suspensions of BCG were revived, many suspensions failing to grow on OAA media that on enrichment yielded a good colony count. Lowenstein–Jensen medium was intermediate between OAA with and without blood, but proved to be too variable from batch to batch to be consistently useful.

Discussion

For the accurate counting of viable organisms the best medium would be one which is simple to make, regularly yields the maximum amount of growth, and is dark in color, thereby facilitating early detection of colonies. The oleic acid albumin medium of Dubos, colored and enriched by the addition of blood, fulfills these requirements.

Although the egg media yielded high counts on occasion, they often gave unpredictably low results. Drea (7) demonstrated that bactericidal substances are readily adsorbed on the surface of glass. The variation in the sensitivity of many media may very well be due to the presence of these substances on routinely cleaned and rinsed glassware, which subsequently contaminate the surface of the medium. This would be especially so in the case of

inspissated egg media, where the condensation of steam on the glass covers of the Petri dishes may well wash off and deposit these substances on the surface of the medium. This variability was never observed with any of the OAA media containing blood. Even in the presence of heavy contamination by bacteria or molds the enriched OAA medium was able to support a considerable growth when no growth was visible on the plain OAA medium.

The growth obtained on blood enriched OAA medium was always more luxuriant than that obtained on other media, and the number of colonies developing was greater at all countable dilutions. Growth was rapid; the colonies were well developed in six to eight days from heavy, and in about ten days from small inocula. Its most striking feature however, was the increased proportion of colonies obtained from reconstituted suspensions of freeze-dried BCG compared with those obtained on other media, as, for example, plain OAA and Lowenstein–Jensen medium.

However, when reconstituted suspensions of BCG were seeded onto solid media there was a longer interval of time before the appearance of microscopic colonies compared with that required for the appearance of colonies from suspensions of fresh BCG, and a more sensitive medium than one yielding a good growth from suspensions of freshly grown BCG was required.

This suggests that the freeze-drying makes a differential change in the viability of organisms surviving the process. Engbaek and Ørskov (11), in a study on the germination and growth of BCG vaccine on Dubos medium, reported the absence of growth of a reconstituted, lyophilized suspension, though good growth was observed from suspensions of either fresh or icebox stored vaccines. In the author's opinion the cause of this change—as yet unknown—may be similar to that of the delay in the conversion of Mantoux-negative patients to Mantoux-positive by freeze-dried and reconstituted BCG vaccine compared with the more rapid conversion by fresh BCG vaccine (1, 2, 3, 23, 25, 29).

Acknowledgments

Grateful acknowledgment and thanks are due to: Dr. R. I. N. Greaves for supervision and advice; to Mr. J. F. Stevenson for help in making the media; and to Mr. Edwards for help with the freeze-drying.

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NOTE ON THE PARASITIC GROWTH-PHASE OF TRICHOPHYTON RUBRUM IN HAIRS[1]

BY F. BLANK[2] AND P. TELNER[3]

Abstract

Hairs removed from the beard of a man with pustular folliculitis were found to be infected by *Trichophyton rubrum*. Three different parasitic patterns could be distinguished: (*a*) the hair was surrounded by a loose network of septate hyphae, (*b*) the hair was encircled by this loose network of septate hyphae and the cuticle invaded by large arthrospores, or (*c*) septate, branching hyphae alone were found inside the hair-shaft. These three endo-ectothrix pictures constitute only transitory forms of the ultimate endothrix parasitic growth-phase of *T. rubrum* which was not reached in this case.

The dermatophytes show a reduced morphology when parasitizing the keratinized parts of hair. However, the morphology and topography of the fungal elements (hyphae and arthrospores) are always so characteristic that a diagnosis of favus, microsporosis, or the various forms of trichophytosis (endothrix, endo-ectothrix-microide, endo-ectothrix-megaspore) can easily be made.

We recently found hairs in which the type of infection did not correspond to any of the above mentioned pictures of parasitism. These grayish, lusterless hairs had been removed from the beard of a man with pustular folliculitis. Almost every second hair removed was found to be affected by fungal elements (hyphae and arthrospores). Three different parasitic patterns could be distinguished. The most often encountered type showed the affected hair surrounded by a loose network of septate hyphae while the hair itself had not been invaded (Fig. 1). The second parasitic picture presented the hair surrounded by the described network of hyphae and the cuticle invaded by large, round arthrospores. However, the arthrospores were found over limited spaces only and never formed a densely packed mosaic or chains of arthrospores as in the typical microsporosis or endo-ectothrix trichophytosis (Fig. 2). Septate, branching hyphae inside the hair-shaft characterized the least often observed third type of infection (Fig. 3). These microscopic findings disclosed very few features similar to recognized parasitic patterns of infected hairs, nor did they allow any speculation regarding the identity of the causative organism.

Trichophyton rubrum (Castellani) Sabouraud, 1911, was repeatedly isolated from inoculated hairs taken from different parts of the patient's beard. *T. rubrum* is primarily an invader of the smooth skin and nails. Invasion of

[1]*Manuscript received October 25, 1955.*
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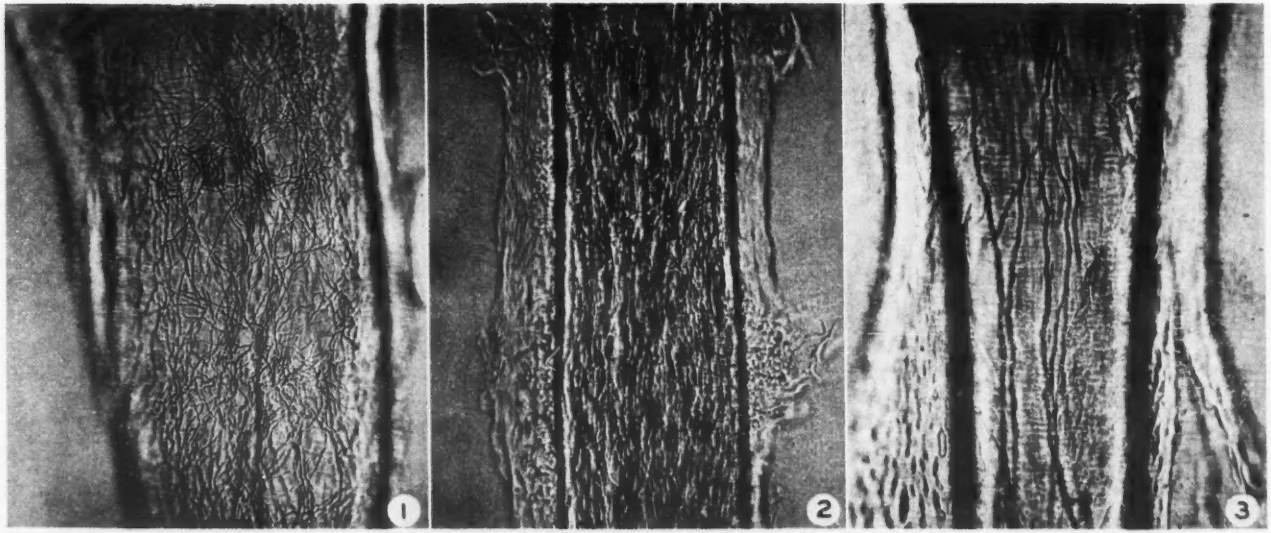

FIG. 1. Hair surrounded by a loose network of septate hyphae.
FIG. 2. Hair surrounded by a loose network of septate hyphae and cuticle invaded by arthrospores.
FIG. 3. Septate, branching hyphae inside the hair-shaft.

the hair by this dermatophyte seems to be rather exceptional. Only an extremely small number of infections of the scalp have been reported (10, 14, 21, 25, 29, 30, 32). The same holds true for infections of the beard; few cases have been reported (2, 11, 12, 15, 16, 17, 18, 19, 20, 28, 31).

Experimental infection of animals from cultures is difficult; only in rare instances have certain strains invaded the animal hair; however, only recently Chakraborty, Ghosh, and Blank (6) isolated *T. rubrum* from animals in India. Although the infection of hairs by *T. rubrum* is extremely rare as compared with the frequency of isolations from skin and nails, there does not seem to exist a group of dermatophytes only attacking the skin and another one only invading hairs, a point already stressed by Payenneville and Rivalier (24).

The reports on the parasitic form observed in hairs infected by *T. rubrum* vary. Kato (10), Milochevitch (20), and Vanbreuseghem (30) found in their respective cases an endothrix pattern. Many have reported an endo-ectothrix type of infection (2, 8, 13, 15, 17, 19, 21, 28, 32). The same conflicting observations were made by those authors who were able to transmit *T. rubrum* from cultures to laboratory animals. Catanei and Lefranc (5), Fujii (7), Hashimoto, Irizawa, and Ota (9), Milochevitch (20), Morikawa (22), and Vanbreuseghem (30) observed an endothrix pattern while Bang (1) and Catanei (3, 4) reported an endo-ectothrix parasitism in their experimentally infected animal hairs.

Although *T. rubrum* is a polymorphous species (23) this fact could not account for the three different parasitic aspects of hairs infected by this dermatophyte which are not comparable to the infection type of any other dermatophyte. Sabouraud (27) and Rivalier (26) have emphasized that an endothrix infection of the hair is always preceded by an endo-ectothrix form as the beginning and transitory stage of hair invasion. Milochevitch (20) was able to infect guinea pigs with a strain of *T. rubrum* that he isolated from a case of sycosis barbae. This author found the same three parasitic patterns among the guinea pig hairs which we observed in our case of sycosis barbae. In addition, he found that the hyphae inside the hair-shaft of the guinea pigs were finally transformed into chains of arthrospores leading to a pure endothrix parasitism. Similar observations were made by Catanei and Lefranc (5) and Hashimoto, Irizawa, and Ota (9). All these findings indicate that the endo-ectothrix parasitism of hairs infected by *T. rubrum* constitutes only a transitory parasitic form which under certain circumstances had not yet reached the final endothrix form. In our case, shaving probably prevented the establishment of the ultimate endothrix pattern of *T. rubrum* infection.

Although very few cases of infection of the scalp by this organism have been reported, it is significant that the majority of these were endothrix infections. In view of these reports and this most recent evidence of the progressive stages of *T. rubrum* invasion of the hairs, any statement that sycosis barbae caused by this dermatophyte is always of the endo-ectothrix type (13) should not overlook the probability that this might be merely the initial stage of an infection which is finally endothrix.

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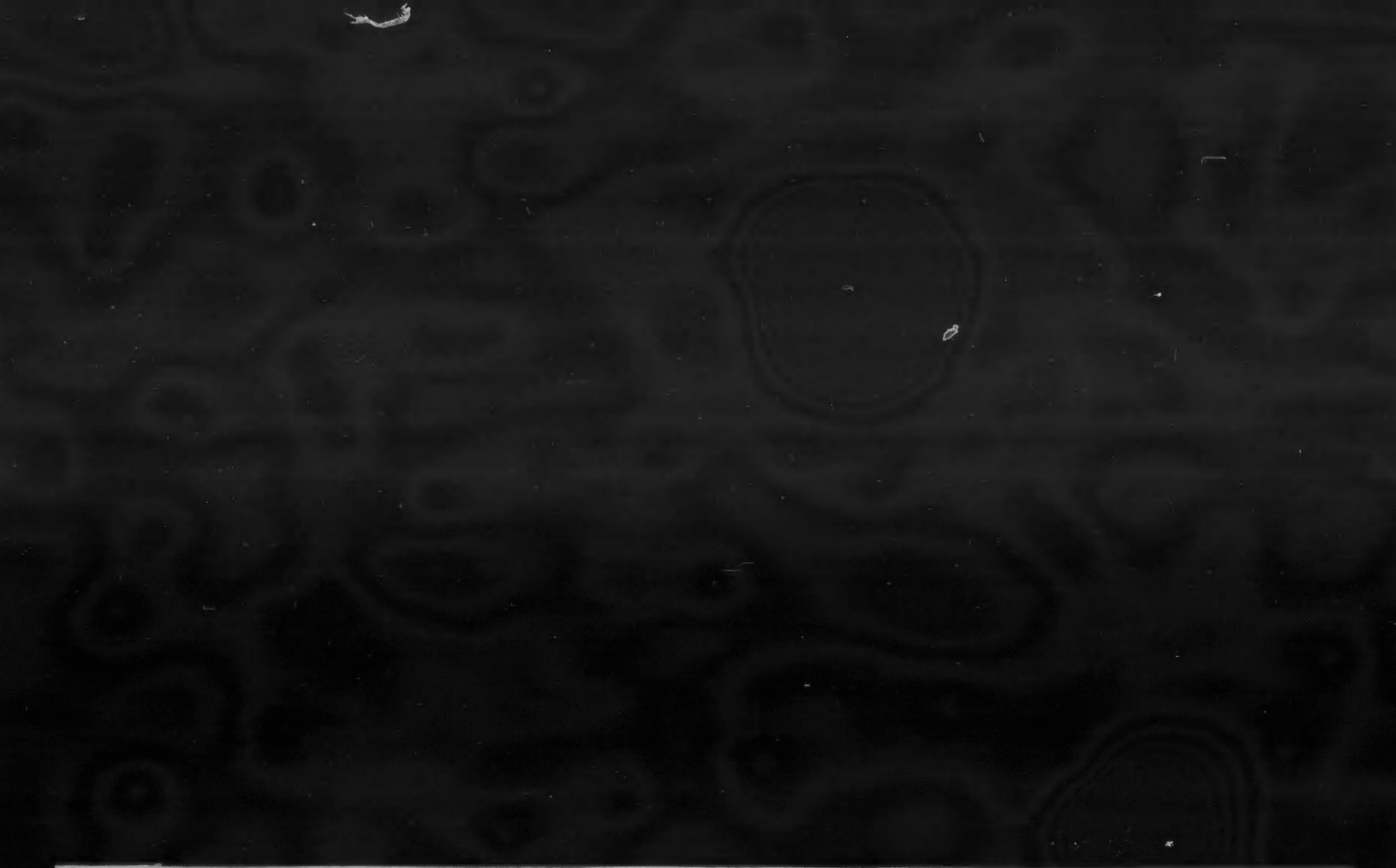
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